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Investigation of the immunomodulatory effects of Antimicrobial Peptides on chicken hepatic cell cultures and intestinal explants

Ph.D. thesis

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1 List of abbreviations

- AMP: antimicrobial peptide
- AMR: antimicrobial resistance
- APC: antigen-presenting cell
- BSA: bovine serum albumin
- Cath-2: chicken cathelicidin-2
- CCK-8: cell counting-kit 8
- CXCLi2: chicken chemotactic and angiogenic factor
- DAMP: damage associated molecular pattern
- DNA: deoxyribonucleic acid
- EGTA: ethylene-glycol tetra-acetic acid
- FBS: fetal bovine serum
- GALT: gut-associated lymphoid tissue
- GI: gastrointestinal
- HBSS: Hanks' Balanced Salt Solution
- HDP: host defense peptide
- H&E: hematoxylin and eosin
- hEGF: human Epidermal Growth Factor
- IDR: innate defense regulator
- IEC: intestinal epithelial cell
- IFN: interferon
- Ig: Immunoglobulin
- IL: interleukin
- LDH: lactate dehydrogenase
- LPS: lipopolysaccharide
- LTA: lipoteichoic acid
- MAPK: mitogen-activated protein kinase
- MDA: malondialdehyde
- M-CSF: macrophage colony-stimulating factor
- M-PER: Mammalian Protein Extraction Reagent
- NADH: nicotinamide adenine dinucleotide hydrogen
- NADPH: nicotinamide adenine dinucleotide phosphate hydrogen
- NF-κβ: nuclear factor-κβ
- NK cells: natural killer cells

- NLRP-3: NOD-, LRR- and pyrin domain-containing protein 3
- Nrf2: Nuclear factor erythroid 2-related factor 2
- PAMP: pathogen associated molecular pattern
- PC: protein carbonyl
- PKC: protein kinase C
- PMA: phorbol myristate acetate
- PRR: pattern recognition receptor
- RANTES: Regulated And Normal T-cell Expressed and Secreted
- (m)RNA: (messenger) ribonucleic acid
- RNS: reactive nitrogen species
- ROS: reactive oxygen species
- TLR: Toll-like receptor

2 Summary

As the threat posed by antimicrobial resistance grows more crucial, the development of compounds that can replace antibiotics becomes increasingly vital. Antimicrobial Peptides (AMPs) provide a feasible solution for the treatment of several inflammatory and infectious diseases among poultry. They are short peptides with a maximum of 100 amino acids that have a wide range of antibacterial properties. However, even more significant are their immunomodulatory properties, which allow them to support the body's immune system in its defense against infections.

Our main was to investigate the effects of two AMPs – one of natural origin and a synthetic one – chicken cathelicidin-2 (Cath-2) and innate defense regulator (IDR)-1002 on cell viability and immune response. Two *in vitro* models were employed for this purpose: a primary hepatocyte – non-parenchymal cell co-culture and an intestinal explant model, both of chicken origin. The reason behind choosing these models was that most of the infections affecting chickens originate from the intestines and are often caused by pathogenic bacteria or by an imbalance in the gut bacteriome. Therefore, it is beneficial to investigate the effects, these promising compounds have on the immune response of the intestinal wall. Moreover, the liver is the first organ to interact with molecules entering the bloodstream from the intestines, therefore it has an especially important role in the protection against infections and pro-inflammatory agents from this direction.

Our first goal was to create a reproducible and reliable inflammatory model using the hepatic cell culture that our research team had previously developed. In this Preliminary study, several pathogen associated molecular patterns (PAMPs) were tested on the hepatocyte – non-parenchymal cell co-culture for their ability to induce inflammation and to monitor their cytotoxicity. From these results, phorbol myristate acetate (PMA) and lipoteichoic acid (LTA) were proven to have a favorable inflammatory effect on our cell cultures.

This was followed by investigating the immunomodulatory effects of the two AMPs, Cath-2 and IDR-1002 on the hepatocyte - non-parenchymal cell co-cultures of chicken origin. Similarly, the peptides were applied to chicken intestinal explant cultures as well to observe their effect on the immune response of the gut wall.

All studies included the measurement of cell viability parameters, as it is inevitably important to determine the possible cytotoxic effects of the compounds, and the potential future *in vivo* use of AMPs requires knowledge on the correlation between different concentrations and possible harmful effects on cells. For monitoring viability, two types of tests were employed: cell counting-kit (CCK)-8 test and extracellular lactate-dehydrogenase (LDH) test, from which

the former measures the metabolic activity of the cells while the latter estimates the integrity of the cell membrane. Furthermore, several inflammatory and redox parameters were measured during our studies, to obtain a broader picture of the immunomodulatory effects of these peptides.

Study I: In the study where Cath-2 was tested on the hepatocyte - non-parenchymal cell co-cultures, both concentrations of the peptide decreased the cellular metabolic activity and increased the LDH activity reflecting a reduction in membrane integrity. Neither LTA nor PMA alone affected these parameters, and when combined with LTA, Cath-2 did not influence the LDH activity. Cath-2 was associated with an increase on the concentration of the proinflammatory chicken chemotactic and angiogenic factor (CXCLi2) and interferon- (IFN-)γ, and the anti-inflammatory interleukin (IL)-10. Meanwhile, macrophage colony stimulating factor (M-CSF), playing a complex role in inflammation, was diminished by the AMP. LTA elevated IFN-γ and decreased M-CSF levels, while PMA only increased the concentration of M-CSF. Both concentrations of Cath-2 increased the H_2O_2 released by the cells, however the concentration of malondialdehyde as a lipid peroxidation marker was not affected. Our findings raise evidence for an anti-inflammatory effect of Cath-2, reflected by the alleviation of the LTA-triggered IFN-γ elevation, and the reduction of the M-CSF production induced by PMA.

Study II: The effects of IDR-1002 were investigated on the same cell culture model. This peptide increased the levels of both RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) and M-CSF, suggesting that it could influence macrophage differentiation, which was further confirmed by the reduced concentrations of IL-6 and IL-10. The pro-inflammatory cytokine release, triggered by the bacterial cell wall component LTA, was ameliorated by the concomitantly applied IDR-1002 based on the levels of IL-6, CXCLi2 and IFN-γ. Moreover, the production of nuclear factor erythroid 2-related factor 2 (Nrf2), an essential transcription factor in the antioxidant defense pathway, was increased after IDR-1002 exposure, while protein carbonyl (PC) levels were also elevated.

Study III: Cath-2 was tested on intestinal explants, where it displayed a potent anti-inflammatory effect suggested by the alleviation of the LTA-caused elevation of IL-6 and IL-2 concentrations, and that of the IFN-γ/IL-10 ratio. Furthermore, it increased the concentration of IL-10, mitigating the LTA-evoked decrease of this anti-inflammatory cytokine. Moreover, when applied alone, an elevation was found in the concentration of IL-6, CXCLi2, and IL-2, providing evidence of the complexity of its immunomodulatory properties.

Study IV: According to the results obtained from testing IDR-1002 on our intestinal explant models, it was found to be non-cytotoxic, and alleviated the LTA-triggered elevation of IL-2, IL-8, RANTES levels, and the ratio of IFN-γ/IL-10, confirming its anti-inflammatory properties. However, immunostimulatory activities could also be observed indicated by the increase in IL-2 and IL-8 concentrations after solitary treatment with IDR-1002.

Our results have shown a notable difference in the cytotoxic potential of these peptides – Cath-2 had more potentially toxic effects than IDR-1002, which aligns with the data concerning the membrane-damaging effects of Cath-2. Furthermore, the hepatocyte - non-parenchymal cell co-cultures seemed to be more sensitive to the effects of this peptide.

Both peptides, Cath-2 and IDR-1002 were shown to have diverse and significant immunomodulatory properties on the models employed in the above studies. Furthermore, our results suggest that the peptides do not have a solely anti-inflammatory, but more of an immunomodulatory effect, which could designate them to help the immune system fight against infections, or control different types of inflammatory diseases. All these properties make them suitable for supplementing the treatment of intestinal infections and perhaps even subclinical chronic diseases affecting chicken production parameters. While there is a certain requirement for further studies to have a more precise understanding of these peptides' mechanisms of action before they can be applied in clinical settings, our results still provide valuable and essential information about their impact on the immune response of the chicken gut and liver.

Összefoglalás

Az antimikrobiális rezisztencia egyre nagyobb fokú terjedésével növekszik az igény az antibiotikum-alternatívák fejlesztésére. Az antimikrobiális peptidek (AMP-k), amelyek kevés, legfeljebb száz aminosavból álló, antimikrobiális és immunmoduláns hatással bíró peptidek, alkalmas jelöltek lehetnek a baromfi különféle fertőző és gyulladásos betegségeinek kezelésére.

Kutatásunk célja kétféle AMP, a természetes eredetű csirke cathelicidin-2 (Cath-2) és a szintetikus „innate defense regulator" (IDR)-1002 sejtek életképességére és immunfunkcióira kifejtett hatásainak vizsgálata volt. Ennek céljából kétféle csirke eredetű *in vitro* modellt alkalmaztunk – egy hepatocita – nem-parenchimális sejt ko-kultúrát és egy bélhám eredetű explant modellt. E sejtmodellek kiválasztásának oka, hogy a csirkéket érintő fertőzések többsége intesztinális eredetű, és gyakran kórokozó baktériumok vagy a bél mikrobiom egyensúlyának felborulása okozza, tehát a vizsgálni kívánt vegyületek bélfal immunválaszára kifejtett hatásának tanulmányozása elengedhetetlen. A máj pedig az első szerv, amely kölcsönhatásba lép a bélből a véráramba kerülő molekulákkal, ezért különösen fontos szerepe van az ebből az irányból érkező fertőzések és gyulladáskeltő anyagok elleni védelemben.

Célunk mindenekelőtt egy reprodukálható és megbízható gyulladásos modell létrehozása volt a kutatócsoportunk által korábban kifejlesztett hepatocita – nem-parenchimális sejt ko-kultúra segítségével. Az előzetes vizsgálatban számos patogén-asszociált molekuláris mintát (PAMP) teszteltünk e ko-kultúrán a gyulladáskeltő képességük és citotoxicitásuk szempontjából. Az eredmények alapján bebizonyosodott, hogy a forbol-mirisztát-acetát (PMA) és a lipoteichosav (LTA) gyulladáskeltő hatása megfelelő az általunk kifejlesztett modellek esetében.

Ezt követte a két AMP, a Cath-2 és az IDR-1002 immunmoduláló hatásának vizsgálata a csirke eredetű hepatocita – nem-parenchimális sejt ko-kultúrán. Hasonlóképpen, a peptideket csirke bélhám explant modellen is alkalmaztuk, hogy pontos képet kaphassunk a bélfal immunválaszára gyakorolt hatásukról.

Vizsgálataink során kétféle, sejtek életképességére vonatkozó paramétert vettünk figyelembe, ugyanis elkerülhetetlenül fontos a vegyületek potenciális citotoxikus hatásainak feltérképezése. Valamint az AMP-k lehetséges jövőbeli *in vivo* felhasználása megköveteli a különböző koncentrációk és a sejtekre gyakorolt esetleges káros hatások közötti összefüggés ismeretét. Az életképesség nyomonkövetésére kétféle tesztet alkalmaztunk: a „cell counting-kit" (CCK)-8 tesztet és a laktát-dehidrogenáz (LDH) extracelluláris aktivitásának mérését, amelyek közül az előbbi a sejtek metabolikus aktivitását, az utóbbi pedig a sejtmembrán integritását vizsgálja. Kutatásunk során továbbá számos gyulladásos és redox paramétert is

mértünk annak érdekében, hogy minél szélesebb képet kapjunk a peptidek immunmoduláló hatásairól.

Az első kísérletünkben a Cath-2 hatásait hepatocita – nem-parenchimális sejt ko-kultúrán vizsgáltuk. Ebben az esetben a peptid mindkét koncentrációja csökkentette a sejtek metabolikus aktivitását és növelte az LDH-aktivitást, ami a membrán integritásának sérülését tükrözi. Sem az LTA, sem a PMA önmagában nem befolyásolta ezeket a paramétereket, LTA-val kombinálva pedig a Cath-2 nem befolyásolta az LDH-aktivitást. A Cath-2 a proinflammatorikus csirke kemotaktikus és angiogén faktor (CXCLi2) és az interferon- (IFN-)γ, valamint a gyulladáscsökkentő hatású IL-10 koncentrációjának növekedését okozta. Eközben a gyulladásban komplex szerepet játszó makrofág kolónia stimuláló faktor (M-CSF) koncentrációja az AMP hatására csökkent. Az LTA megemelte az IFN-γ és csökkentette az M-CSF szintjét, míg a PMA csak az M-CSF koncentrációját növelte. Ezzel szemben, a gyulladáskeltő vegyületek által megemelt IFN-γ és M-CSF szinteket az egyidejűleg alkalmazott Cath-2 csökkenteni tudta, mely eredmények alapján megállapíthatjuk, hogy a Cath-2 gyulladáscsökkentő hatással rendelkezik. A Cath-2 mindkét koncentrációja növelte a sejtek által felszabaduló H_2O_2-t, azonban a lipidperoxidációt jelző malondialdehid koncentrációját nem befolyásolta.

Második kísérletünkben az IDR-1002 hatását ugyanezen a sejtkultúra-modellen vizsgáltuk. A peptid megnövelte mind a RANTES (Regulated on Activation, Normal T cell Expressed and Secreted), mind az M-CSF szintjét, ami arra utal, hogy befolyásolhatja a makrofágok differenciálódását, amit az IL-6 és IL-10 koncentrációjának csökkenése is megerősített. Az LTA által kiváltott pro-inflammatorikus citokin felszabadulást az IL-6, CXCLi2 és IFN-γ mért értékei szerint az egyidejűleg alkalmazott IDR-1002 mérsékelte. Ezenkívül az antioxidáns védekezési útvonalban alapvető fontosságú transzkripciós faktor, a nukleáris erythroid 2 faktor 2 (Nrf2) termelése megnövekedett az IDR-1002 expozíciót követően, miközben a protein karbonil (PC) szint is emelkedést mutatott.

Harmadik kísérletünkben a Cath-2 vizsgálata bélhám eredetű explantok segítségével történt. Az AMP ebben az esetben kifejezett gyulladáscsökkentő hatást mutatott, amire az IL-6 és IL-2 koncentráció LTA okozta emelkedésének mérsékléséből, valamint az IFN-γ/IL-10 arányának csökkentéséből tudunk következtetni. Továbbá a peptiddel való kezelés következtében megfigyeltük a gyulladáscsökkentő hatású IL-10 koncentrációjának emelkedését, mérsékelve a citokin LTA által kiváltott csökkenését. Valamint a Cath-2-t önmagában alkalmazva az IL-6, a CXCLi2 és az IL-2 koncentrációjának emelkedését tapasztaltuk, ami bizonyítékot szolgáltat immunmoduláló tulajdonságainak összetettségére.

Negyedik kísérletünkben az IDR-1002-t vizsgáltuk bélhám eredetű explant modelljeinken. Eredményeink alapján a peptid nem volt citotoxikus, és enyhítette az IL-2, IL-8, RANTES szintjének és az IFN-γ/IL-10 arányának LTA által kiváltott emelkedését, ami

gyulladáscsökkentő tulajdonságára enged következtetni. Ugyanakkor immunstimuláló hatás is megfigyelhető volt, amire az IL-2 és IL-8 koncentráció növekedése utal az IDR-1002-vel történő kezelést követően.

Eredményeink alapján jelentős különbség figyelhető meg a különböző peptidek citotoxikus hatásaiban – a Cath-2 potenciálisan toxikusabb hatást fejtett ki, mint az IDR-1002, ami összhangban van a Cath-2 membránkárosító hatásaira vonatkozó adatokkal. Továbbá a hepatocita – nem parenchimális sejt ko-kultúrák érzékenyebbnek bizonyultak e peptid hatásaira.

A Cath-2 és az IDR-1002 peptid egyaránt változatos és jelentős immunmoduláló tulajdonságokat mutatott az általunk végzett vizsgálatokban alkalmazott modellekben. Eredményeink arra utalnak, hogy ezen AMP-k gyulladáscsökkentő tulajdonságuk mellett jelentős komplex immunmoduláló hatásokkal rendelkeznek, ami alkalmassá teheti őket az immunrendszer fertőzések elleni küzdelemben való hatékony segítésében, illetve a különböző típusú gyulladásos betegségek kezelésében. Mindezek a tulajdonságok alkalmassá tehetik őket az intesztinális eredetű fertőzések, valamint a baromfiban előforduló, termelési paramétereket negatívan befolyásoló szubklinikai, krónikus betegségek kezelésének kiegészítésére. Bár pontos hatásmechanizmusuk feltérképezéséhez további vizsgálatok szükségesek esetleges klinikai körülmények között történő alkalmazásuk előtt, eredményeink mégis értékes és lényeges információkkal szolgálnak a csirke bélrendszerének és májának immunválaszára gyakorolt hatásaikról.

3 Introduction and literature overview

3.1 Introduction

Poultry, the largest meat-producing livestock species kept today, are highly exposed to various infectious diseases due to crowded housing conditions, which can be exacerbated by stressors such as heat or transportation. Some of these diseases are of bacterial origin and are proving increasingly difficult to treat since the spread of antimicrobial resistance (AMR). Antibiotics are becoming less and less effective and the use of agents to which pathogens are still susceptible is increasingly restricted by the need to protect human life. The demand for natural and synthetic substitutes for antibiotics is therefore growing.

Living organisms have various ways to defend themselves against harmful effects, for example by secreting biologically active substances. Antimicrobial peptides (AMPs), also known as host defense peptides (HDPs) are an important part of this system, produced by almost all organisms, and are promising candidates in the treatment of bacterial and inflammatory diseases. They are composed of up to 100 amino acids and are considered to have a direct antimicrobial effect as part of the innate immune system and to be able to modulate the immune response. Although their discovery is mainly due to their direct antimicrobial property, it has subsequently been demonstrated that in vertebrates, including humans, they can protect against pathogens by stimulating the body's self-defense and by suppressing harmful inflammatory processes. Their direct antimicrobial effect is poorly observed *in vivo* which is the reason why the term HDP is nowadays often used in the literature to refer to their protective function in assisting the immune system. To date, more than 3500 AMPs have been described in the University of Nebraska Medical Center database (https://aps.unmc.edu/).

As part of this dissertation, an AMP of natural (chicken cathelicidin-2, Cath-2) and one of synthetic origin (innate defense regulator-1002, IDR-1002) were investigated. Naturally derived AMPs have a larger history of literature and therefore more information is available about them, but they have several disadvantages compared to synthetic AMPs, such as higher levels of toxicity and species specificity. However, synthetic AMPs have been developed considering aspects such as smaller size and increased potency.

3.2 Antimicrobial Peptides

3.2.1 History

In 1926, the first evidence of a naturally occurring antimicrobial molecule was found in flour, as it was discovered to contain a substance that can inhibit the growth of mold on bread [1]. However, the molecule itself was not identified until 1942, when a protein capable of inhibiting the growth of various phytopathogenic bacteria and mold species was detected in the endosperm of wheat [2]. This protein was then named purothionin in the 1970s. In later years, several plant-derived AMPs belonging to the thionin group have been discovered [3].

The therapeutic use of penicillin (discovered in 1928 by Alexander Fleming) began in the 1940s [4], marking the golden age of antibiotics and shifting focus away from research on other antimicrobial agents. In 1939, between the discovery and clinical application of penicillin, the first AMP was described by Dubos et al, when an extract with antimicrobial activity was isolated from a *Bacillus brevis* strain in soil, which proved to be effective against *Pneumococcus* infection in mice [5].

A year later, Hotchkiss and Dubos successfully isolated the active molecule from the extract, naming it gramicidin [6]. Although it seemed to exert some toxic effects systematically, it aided in wound and ulcer healing when applied externally, therefore clinical use soon became available [7]. This was followed by Dubos and Hotchkiss' discovery of tyrocidin in 1941 [8], and the identification of the aforementioned purothionin in 1942.

In the 1960s, the emergence of multi-resistant bacteria [9] renewed the interest in molecules with antimicrobial properties. Consequently, researchers redirected their focus towards antimicrobial peptides, leading to the discovery of numerous molecules with protective functions in the following decades [10]. During the 1950s and 1960s, several research groups observed that white blood cells in various species, including rabbits, humans, and guinea pigs, could produce cationic proteins with antimicrobial properties, seemingly independent of adaptive immunity [11]. Bombinin, often regarded as the first animal-derived AMP, was isolated in 1962 from the skin secretions of *Bombina variegata* toads [12]. Since then, various bombinin-like peptides have been identified from this and other species within the *Bombina* genus [13]. Lactoferrin (or lactotransferrin), a protein with iron-binding abilities, was initially isolated from cow's milk by Sorensen et al. in 1939 [14]. However, its antimicrobial activity was only recognized after its discovery in breast milk during the 1960s [15–18]. Concurrently, researchers found that immunizing wax moth larvae against *Pseudomonas aeruginosa* prompted the production of a bactericidal protein targeting this pathogen [19].

In the 1970s and 1980s, several independent research groups identified various AMPs from human and rabbit white blood cells [20], including lactoferrin [21], bactericidal/permeability-increasing protein [22], defensins [23], and serine protease homologues [24]. In 1981, Boman and colleagues induced the production of alpha-helical cationic peptides in the larvae of the silkworm (*Hyalophora cecropia*), later named cecropins [25, 26]. Additionally, in 1987, Zasloff and co-workers isolated AMPs from African clawed frogs, and named them magainin from the Hebrew word for shield [27].

During the 1990s, the idea that AMPs might play a crucial role in the defense of organisms lacking adaptive immunity gained attention [28]. This hypothesis was supported by the studies on *Drosophila melanogaster*, where the deletion of an AMP encoding gene resulted in diminished defense against fungal infections [29]. In recent years, numerous AMPs have been discovered in invertebrates, particularly from the skin secretions of various amphibians. The dermaseptins, produced by tree frogs, represent the largest group, with hundreds of members identified to date [30–32]. Additionally, several plant-derived antibacterial and antifungal molecules have been isolated [33]. The search for novel AMPs continues unchanged, with recent efforts focusing on the production of synthetic peptides engineered to minimize cytotoxicity while maximizing efficacy.

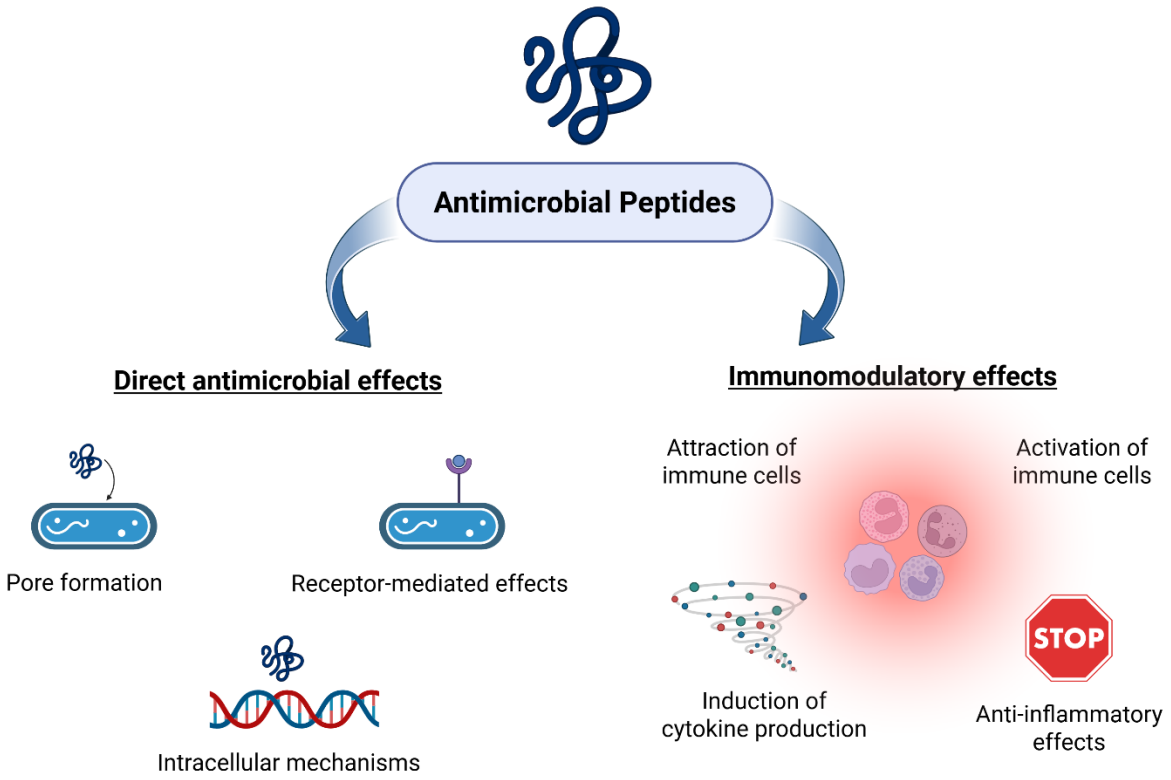

Figure 1. The multifaceted effects of Antimicrobial Peptides. Own figure, created with BioRender.com.

3.2.2 Antimicrobial mechanisms

The antimicrobial effects of AMPs can be categorized into two primary mechanisms: direct killing and immunomodulation (see **Figure 1**). The direct killing mechanism is mainly attributed to their cationic nature, primarily presented by lysine, arginine, and histidine residues. Moreover, the hydrophobicity of these peptides, typically facilitated by amino acids such as valine, leucine, isoleucine, alanine, methionine, phenylalanine, tyrosine, and tryptophan is crucial for bacterial membrane permeabilization [34]. Additionally, the amphipathicity of AMPs, which includes a balance between cationic hydrophilic and hydrophobic molecular segments, also plays a significant role [35]. These properties - peptide charge, hydrophobicity, and amphipathicity - collectively determine the antibacterial activity and cytotoxicity of each AMP, contributing to their unique characteristics.

The direct antibacterial action of AMPs can be divided into two processes: attachment to the cell membrane with pore formation, and subsequent action within the cell cytoplasm or nucleus [36]. While the speed and precise mechanism of bactericidal action may vary for each peptide, there are identifiable steps and processes that generally describe the action of AMPs [37].

Cationic peptides have an affinity for the outer surface of bacteria, which tends to be more negatively charged due to the presence of outwardly directed anionic "head" groups of phospholipids. Additionally, in Gram-negative bacteria, abundant lipopolysaccharide (LPS), and in Gram-positive bacteria, lipoteichoic acid (LTA) molecules contribute to this negative charge [38]. To initiate pore formation, peptides must accumulate at a certain concentration on the bacterial membrane, typically in the micromolar range [35].

The pore formation mechanism of AMPs can be explained by three models (see **Figure 2**). One of these is the barrel-stave model, observed in relatively few AMPs. In this case, peptides with α-helix or β-edge structures initially align parallel to the membrane. Upon reaching the required concentration, they form a barrel-shaped pore perpendicular to the membrane, with a hydrophobic membrane-facing portion and a hydrophilic luminal section [39, 40]. Another model is the "toroidal pore" model, analogous to the barrel-stave model but with fewer peptide-peptide interactions and more peptide-membrane interactions within the pore. In this model, the membrane recoils to form part of the pore, with the head portions of phospholipid molecules facing the interior of the pore [40]. The "carpet model" offers a different approach, where AMPs do not form pores but aggregate parallel to the cell surface. With sufficient peptide accumulation, the cell membrane ruptures due to the detergent-like action of the peptides. In this scenario, no peptide-peptide interactions occur, and no pores are formed in the membrane [41].

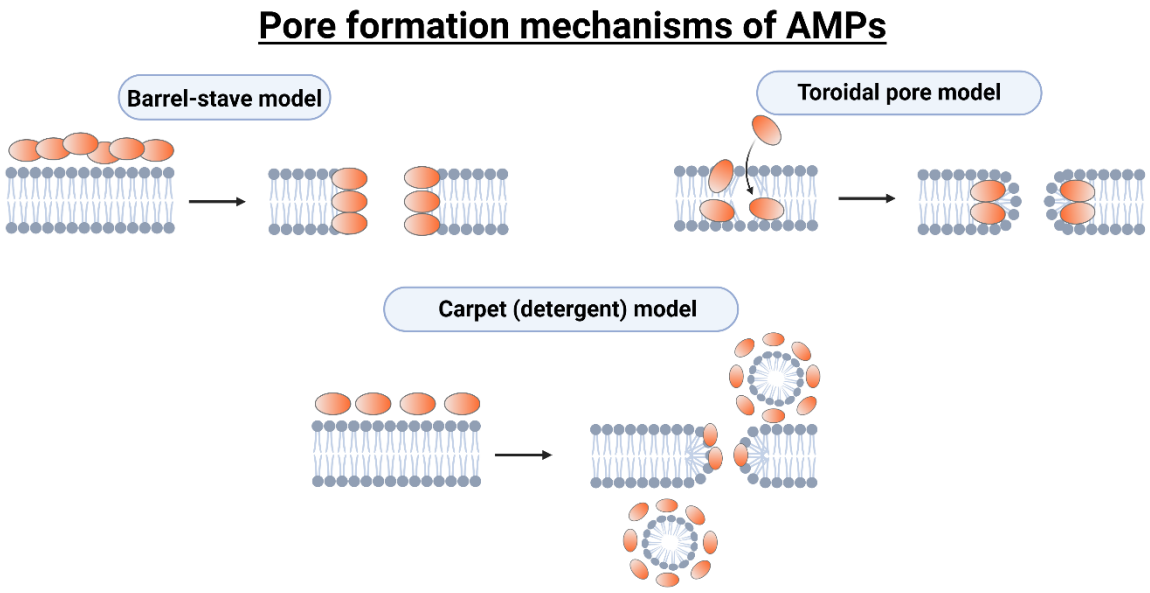

Figure 2. Pore formation models for Antimicrobial Peptides (AMPs): barrel-stave model, toroidal pore model, and carpet (detergent) model. Round substances represent AMP molecules. Own figure, created with BioRender.com.

The generated pores and channels change the transmembrane potential, and this, along with the resulting disruption in permeability, proves sufficient for bacterial destruction in many cases. However, at times, since the channels only remain active for a short period [42], the peptides that enter through them disrupt the function of intracellular polyanionic molecules, such as the deoxyribonucleic acid (DNA) [43], or they can deter the synthesis of proteins and nucleic acids [44, 45]. Moreover, certain peptides can interfere with cell wall synthesis by interacting with specific precursors, such as lipid II molecules [46]. Some AMPs are capable of this even without forming pores in the membranes but by entering cells through alternative means, allowing for bactericidal effects to be achieved with lower concentrations [37]. It is also noteworthy that cationic peptides can potentiate the effects of certain antibiotics by facilitating their entry into bacterial cells through the channels they form [47].

3.2.3 Immunomodulatory properties

AMPs are produced by almost every living organisms – bacteria, plants, insects, invertebrates and vertebrates all use these peptides in their defense system against harmful microorganisms [47]. In humans and mammals, various AMPs are produced by white blood cells and epithelial cells in the skin and gastrointestinal (GI) tract [48]. These peptides can accumulate in high concentrations in specific locations, such as leukocyte granules [49] or intestinal crypts [50]. At such concentrations, they can directly damage bacterial membranes, exerting bactericidal effects. However, these high concentrations are rarely achieved except in specific instances mentioned above. In most sites, AMP concentrations are significantly lower and may not be directly antimicrobial due to the presence of physiological concentrations of salts, proteins, lipoproteins, and glycosaminoglycans, which can inhibit their action [51]. Nonetheless, even at lower concentrations, AMPs may still inhibit pathogen proliferation. For instance, IDR-1, a synthetic AMP lacking *in vitro* antimicrobial activity, has been shown to protect mice from infection by pathogenic microorganisms [52]. This underscores that the effects of AMPs often involve active and complex manipulation of the immune system rather than direct antimicrobial action.

Their immunomodulatory effects include the activating, attracting, and differentiating effect of various cells, along with the modulation of inflammatory mediator and reactive oxygen/nitrogen species (ROS/RNS) production [53]. Recently, increasing attention has been directed towards these effects of the AMPs. Understanding their role in the immune system in detail is crucial for their successful utilization in treating various diseases (see **Figure 3**).

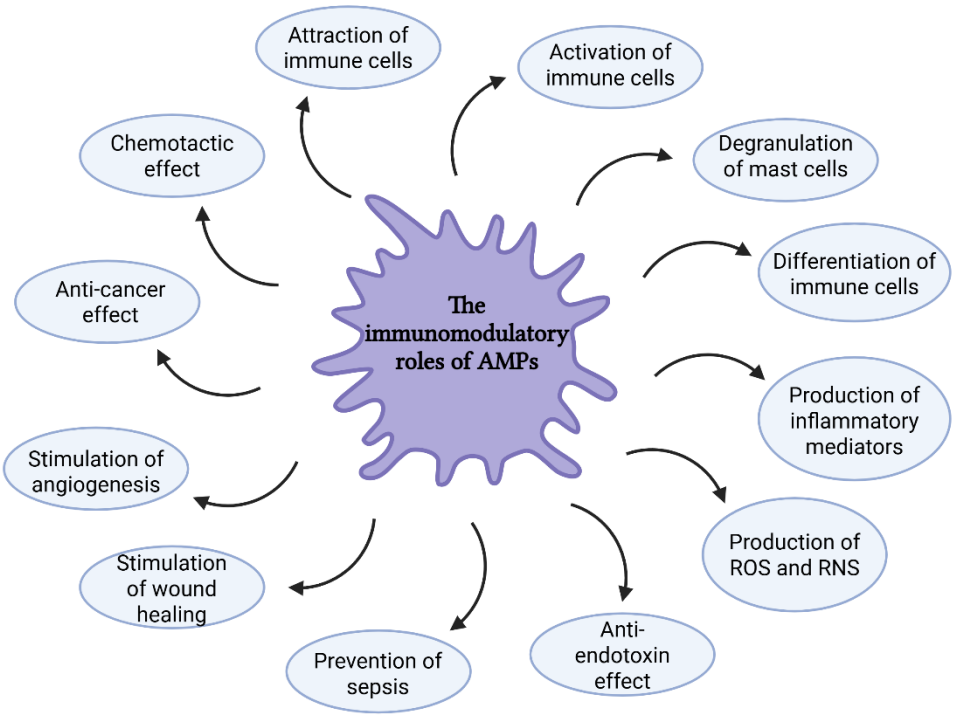

Figure 3. The effects of Antimicrobial Peptides on the immune system. ROS=Reactive oxygen species, RNS=reactive nitrogen species. Own figure, created with BioRender.com.

Several AMPs can be produced and released by white blood cells, mainly neutrophils (or, in the case of birds, heterophils). The degranulation of these cells results in relatively high concentrations of AMPs, which (directly or indirectly) leads to the chemoattraction of different types of immune cells, for example, T-cells, eosinophils, macrophages and mast cells [54]. Direct attraction is mediated by several chemokin receptors, toll-like receptors and G-protein coupled receptors. Furthermore, defensins were shown to attract immune cells not only directly but also by increasing the expression of interleukin-8 (IL-8) in epithelial cells [55]. Additionally, it was demonstrated that AMPs exhibit a wide range of pro- and anti-inflammatory properties toward tissue resident cells as well as recruited leukocytes [51]. These peptides also play an important role in connecting the innate and adaptive immunity, mainly by attracting the different leukocytes to one place and therefore enabling them to interact with one another.

Another aspect of immunomodulation is when the polarization of macrophages is directed either towards the pro-inflammatory M1 or the anti-inflammatory M2 phenotype [56]. Several AMPs demonstrate this effect, for example the humane cathelicidin LL-37, which directs macrophages towards the M1 type [57]. Furthermore, AMPs have the ability to increase the synthesis and change the concentrations of cytokines and chemokines, however the precise mechanism underlying this effect varies greatly depending on the peptide and cannot

be explained generally. They can, however, alter the immune response in a wide range of mechanisms through these signaling substances [51].

3.2.4 Cathelicidins

Cathelicidins, one of the largest groups of naturally occurring AMPs, have a significant impact on innate immune function [51]. Besides epithelial cells in the digestive, respiratory and reproductive tracts, they are mostly expressed in myeloid progenitor cells and their pro-peptides are held in neutrophil granules where they undergo proteolytic cleavage upon degranulation to transform into active forms [58]. Among them, human LL-37, transcribed from the cathelicidin AMP (CAMP) gene, is the best-known and most extensively studied, as it is produced by various immune and epithelial cells [59]. Besides its direct antimicrobial activity, LL-37 exhibits diverse immunomodulatory effects depending on cell type and inflammatory stimuli [60]. Epithelial cells release LL-37 in response to injury or infection [59], with its secretion stimulated by vitamin D3 [61]. The influence of LL-37 on immune cells is complex, as it binds to numerous extracellular and intracellular receptors and capable of inducing hundreds of genes [62]. It also exerts anti-inflammatory effects by promoting the differentiation of macrophages into M1 pro-inflammatory cells, thereby enhancing cellular defense [57]. However, LL-37 can also reduce the production of pro-inflammatory cytokines in response to inflammatory stimuli, mitigating the adverse effects of inflammation [63].

Cathelicidins have well-documented broad-spectrum activity *in vitro*. For example, bovine cathelicidins BMAP-27, BMAP-28 and analogs were found to be effective against a wide range of clinical isolates (including antibiotic-resistant Gram-positive and Gram-negative bacteria) at low micromolar MICs. BMAP-27 and selected analogs were especially potent against Gram-negative bacteria, and BMAP-28 against Gram-positive, and several peptides protected mice in an acute peritonitis model at doses far below toxic levels [64]. Likewise, chicken cathelicidins exhibit robust direct antibacterial effects: all three chicken peptides (CATH-1, -2, -3) tested showed strong killing of multidrug-resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterobacter* and other bacteria, independent of their resistance profiles. Repeated exposure did not induce major resistance to these peptides. [65]. Mechanistic studies confirm rapid bactericidal action; e.g. imaging of chicken CATH-2 showed it binds and permeabilizes *Escherichia coli* membranes within minutes [66]. Similarly, a water-buffalo cathelicidin (WBCATH) killed both drug-sensitive and MDR *Mycobacterium tuberculosis in vitro* (with electron microscopy revealing membrane disruption) and significantly lowered lung bacterial burdens in a mouse tuberculosis model [67].

In chicken, cathelicidins play a crucial role in the fight against infections as part of the innate immune system. There are currently four known cathelicidins in chickens (cathelicidin 1-3 and cathelicidin B1) which are expressed in several types of tissues including the GI, respiratory, and urogenital system, as well as the immune organs including the bone marrow and the bursa of Fabricius [58]. Cath-2 is exclusively produced by heterophils, which are recruited to the site of infection as a key part of the early immune response [68] and then release the peptide from their granules [69–71]. The advantageous effects of Cath-2 have been described in many species, including chicken, human, rodents, and swine [72–77]. It has also been proven to inhibit pathogen associated molecular pattern (PAMP)-induced pro-inflammatory processes, and to enhance the immune protection of the host helping them to fight infectious diseases [71, 78–83].

3.2.5 Innate Defense Regulator peptides

The development of synthetic AMPs with enhanced immunomodulatory capabilities represents a significant advancement. A notable example is the family of IDR peptides, engineered from bactenecin, the shortest naturally occurring cationic AMP found in cattle [84]. These peptides exhibit superior immunomodulatory activity compared to the naturally produced AMPs. Importantly, while they retain their cationic and amphipathic properties, which facilitate membrane interactions, these properties are less pronounced. This results in reduced direct antibacterial activity but also decreases the likelihood of cytotoxic interactions with eukaryotic cell membranes [52]. Presently, numerous studies are focused on exploring their immunomodulatory and anti-infective effects.

The peptide IDR-1 exhibited a unique trait: it lacked direct antimicrobial activity under *in vitro* conditions. However, in experimental mouse models, IDR-1 was demonstrated to suppress both Gram-positive and Gram-negative bacterial infections by modulating the production of various inflammatory mediators [52]. Subsequently, several other IDR peptides were developed, including IDR-HH2, IDR-1002, and IDR-1018, all of which displayed promising results in various infection models involving pathogens such as *Staphylococcus aureus*, *Escherichia coli*, or *Mycobacterium tuberculosis* [53, 85]. Moreover, IDR-1002, for instance, has been shown to induce neutrophil granulocyte migration and cytokine production, as well as facilitate the delivery of other AMPs, such as the neutrophil granulocyte-derived cathelicidin LL-37 [86].

It's worth noting that IDR-1018 possesses the ability to induce macrophages to differentiate into a cell type exhibiting characteristics between the pro-inflammatory M1 and the anti-inflammatory M2 forms [87]. Promising results have also emerged from clinical studies: IDR-1008 demonstrated effectiveness in increasing the survival rate of mice infected

with malaria [88], and IDR-1002, when combined with ciprofloxacin, emerged as a promising candidate for expediting recovery following dental surgery [89].

3.3 Antimicrobial resistance

The first feed additive containing antibiotic substance was approved for poultry in 1948, when sulfaquinoxaline was added to chicken feed to control coccidiosis. By the 1950s, low-dose antibiotics were widely adopted on farms for growth promotion and disease prevention, contributing to an increase in meat production and also creating conditions ideal for the emergence of AMR [90]. Over the next decades, evidence grew that overuse of antibiotics in livestock contributed to AMR in pathogens. This resulted in the European Union banning antibiotic growth promoters in animal feed in 2006 [91]. Similar restrictions and stewardship programs have since been implemented or proposed in many countries. Despite these efforts, antibiotic use in farm animals still accounts for a significant portion of global consumption, and AMR has continued to rise as a pressing One Health issue [92].

Poultry production faces several bacterial diseases that cause considerable economic losses. One of the most important among these is colibacillosis, caused by avian pathogenic *Escherichia coli* (APEC). Colibacillosis can lead to septicemia and organ lesions in chickens, resulting in high mortality and carcass condemnations. It is prevalent worldwide and is responsible for significant economic losses to the poultry industry [93]. Another major disease is necrotic enteritis, an enteric infection of broiler chickens caused by *Clostridium perfringens*. Necrotic enteritis is ubiquitous and costly; it has been estimated to cause around $6 billion in annual losses globally due to reduced growth and increased mortality in flock [94]. Moreover, *Salmonella enterica* serovars such as Pullorum and Gallinarum (which cause Pullorum disease and fowl typhoid) can devastate flocks with high acute mortality. Pullorum disease is considered a serious threat to chicken health worldwide and a source of contamination in poultry products [95]. Additionally, zoonotic serovars like *Salmonella* Typhimurium often infect poultry subclinically, posing food safety risks. Chronic respiratory infections like mycoplasmosis (caused by *Mycoplasma gallisepticum, Mycoplasma synoviae*) are another significant problem: these pathogens lead to airsacculitis, poor weight gain, and drops in egg production, causing substantial economic losses in both meat and egg-laying operations [96]. Other notable bacterial diseases include fowl cholera (*Pasteurella multocida*) and infectious coryza (*Avibacterium paragallinarum*), which can cause high morbidity and losses, especially in the absence of effective antibiotics [97].

To combat these infections, the poultry industry has long relied on various antibiotics. Broad-spectrum antibiotics such as tetracyclines, beta-lactams (e.g. penicillins,

cephalosporins), sulfonamides (often combined with trimethoprim), aminoglycosides (e.g. gentamicin), and fluoroquinolones (e.g. enrofloxacin) are commonly used to treat or prevent bacterial diseases in chickens [98]. Macrolide antibiotics like tylosin are frequently used against *Mycoplasma* infections, and ionophore or polypeptide antibiotics (e.g. bacitracin) have been used as feed additives to control clostridial gut disease. Extensive use of these drugs has led to the emergence of resistant strains in many poultry pathogens. For example, surveys of APEC from commercial chickens show very high resistance rates to first-line antibiotics – in one recent study, over 99% of *Escherichia coli* isolates were resistant to ampicillin, and more than 90% were multi-drug resistant [99]. *Salmonella* from poultry has similarly evolved resistance to critical drugs: the use of fluoroquinolones and third-generation cephalosporins in poultry has selected for ciprofloxacin- and cefotaxime-resistant *Salmonella*, reducing treatment options for both animals and humans [100]. *Mycoplasma gallisepticum* has developed resistance to macrolides in some regions, and *Clostridium perfringens* strains with reduced susceptibility to poultry-used antimicrobials have been reported. The overall pattern is that many poultry bacterial isolates now exhibit resistance to multiple antibiotic classes, undermining the efficacy of traditional treatments [101]. This trend is alarming not only for poultry but also for public health, since resistant poultry pathogens or resistance genes can spread to humans through the food chain.

In response to rising AMR, there is growing interest in alternatives to antibiotics for poultry. One promising strategy is the use of AMPs as feed supplements or therapeutic agents (detailed description of AMPs can be found in the next chapter of this dissertation). A limited number of AMP-based products have already been tested in farm animals, however, specific poultry diseases have been targeted in recent studies to evaluate AMP efficacy. For example, necrotic enteritis control has seen advances using AMPs: in an experimental infection, adding a novel peptide to the feed of broilers significantly reduced *Clostridium perfringens*-induced gut lesions and improved growth performance, performing as well as a conventional antibiotic growth promoter in mitigating necrotic enteritis outcomes. This suggests AMPs could be a viable substitute for antibiotics in preventing clostridial enteritis in chickens [102].

Another case is *Salmonella* Pullorum (fowl typhoid), where a specific designer peptide (named HJH-3) was tested as a therapeutic. In infected chicks, oral administration of HJH-3 protected over 70% of the birds from lethal dose exposure – a survival rate comparable to that achieved with ampicillin treatment. The peptide-treated chickens had lower bacterial counts in organs and milder intestinal lesions, demonstrating that AMPs can prevent and treat systemic *Salmonella* infections in poultry [95]. There is also evidence that dietary AMPs can help control colibacillosis and improve gut health. For instance, feeding broilers a lactoferrin-derived

peptide was shown to reduce intestinal *Escherichia coli* populations while boosting beneficial bacteria, thereby enhancing disease resistance in the flock [92].

These examples illustrate that AMP-based interventions could target a range of poultry pathogens – from gut bacteria causing enteritis to respiratory or systemic infections – offering an alternative to conventional antibiotics. Although challenges remain (such as cost of production and stability of peptides in feed), the continued development of AMPs and other feed additives is a critical part of reducing antibiotic use and combating antimicrobial resistance in the farm industry.

3.4 Anatomy and immune system of the gut and liver of chickens

Chickens held in high-density farming environments are at increased risk of infections, especially of GI origin. Their GI tract is exposed to several negative impacts, including rapid growth, crowded housing, possible stress caused by heat or transportation, and various pathogenic microbes [103]. The maintenance of gut health is of great importance as the GI mucosa is the first line of defense to interact with pathogenic agents entering the body, therefore, it has a rather complex role in regulating the differentiation between neutral and harmful molecules. Gut health is defined as the adequate function of four major components: nutrition, microbiome, GI mucosa and immune system [104, 105].

The GI tract of chickens has unique anatomical, histological, and physiological characteristics. It is relatively shorter than in most mammals, therefore, it has a lesser retention time [106], which makes it even more important to keep its functions at the highest possible level. Their keratinized tongue acts as a first line of defense, as besides the physical barrier it provides, it can produce several AMPs, for example, defensins and gallinacins [107]. Through the esophagus, the food first enters the crop which is an important site for fermentation as a temporary storage with several types of microorganisms living there [107] and also provides protection through the production of β-defensin AMPs [108]. Then the food travels to the proventriculus and the muscular stomach (the gizzard). These two both produce digestive enzymes and HCl, which leads to lower pH levels. Besides that, the proventriculus also provides a mucus barrier consisting of complex carbohydrates, spasmolytic polypeptide [109], and AMPs, for example, β-defensin-6 [108].

The small intestine (duodenum, jejunum and ileum) has a complex function in the digestion and the protection against foreign substances. Its outer layer with the finger-like villi and the glandular crypts provides four types of barriers: microbial, chemical, physical and immunological [110]. The chemical consists of the production of digestive enzymes, and the

physical is provided by the mucus (mucopolysaccharide) layer and the tight junction between the epithelial cells [106]. The outer layer of the mucus provides a habitat and a source of nutrients for commensal bacteria, while inhibiting the growth of pathogenic microorganism [111]. Meanwhile, the inner layer acts as a “protected zone”, as it separates the content of the intestinal lumen from the host. It produces several types of AMPs, immunoglobulin A (IgA), and other bioactive molecules [112].

Compared to mammals, chickens have shorter large intestines. The colon's structure is similar to that of the small intestine, and it has an essential role in the metabolism of nitrogen and the absorption of water. In addition to being an important site for fermentation and water absorption, the caecum also produces a significant quantity of vitamins and volatile fatty acids from the fermented fibers. [107].

Since several kinds of antigens from diet and the microbiome of the gut constantly challenge the immune system, inflammation, which is an essential component of both innate and adaptive immune responses, plays a crucial role in the protection of the host from pathogen microorganisms. Besides, it also has an important part in the healing processes following damage to the epithelium and also in the maintenance of gut health. Therefore, to maintain the balance between pro- and anti-inflammatory processes is of great importance, as it is crucial for the well-being of the animal [106]. In the case of an infection, PAMPs are recognized by various Toll-like receptors (TLRs, for example TLR1, 2, 3, 4, 5, 7 and 15 [78]) expressed on the surface of around 20 types of epithelial and immune cells [113]. The activation of these receptors results in the induction of signaling cascades, such as the mitogen-activated protein kinase (MAPK) and the nuclear factor-κβ (NF-κβ) pathways. As a consequence, several different cytokines, chemokines and even AMPs will be produced [78].

The cells responsible for the immune system of the gut are part of the gut-associated lymphoid tissue (GALT). There are several diffusely located cells and some organized lymphoid structures as well. The latter are the bursa of Fabricius, the cecal tonsils, the Peyer’s patches, the Meckel’s diverticulum, and the lymphocyte follicles in the lamina propria layer of the epithelium. It is important to note that chickens do not have the same lymph nodes as mammals, instead they have lymphoid aggregates. These are mostly consisted of heterophils, macrophages, dendritic cells, natural killer (NK) cells, B and T cells.

B cell development takes place in the bursa of Fabricius, a primary lymphoid organ which is only found in avian species. The Peyer’s patches are secondary lymphoid tissues and are located in the *lamina propria* and the submucosa of the distal ileum. Together with the cecal tonsils, they play an essential role in regulating the immune response to pathogens entering the intestine as they help to facilitate the sampling of the luminal contents and the delivery of the antigens to the antigen-presenting cells (APCs), such as dendritic cells and macrophages

[114]. Although the exact function of the Meckel's diverticulum is still unknown, it contains many macrophages and B cells providing help in the immune protection of the gut [115].

Besides absorption and digestion, intestinal epithelial cells (IECs) also play an especially important part in immune protection. The enterocytes form a physical barrier, the goblet cells secrete the mucin and Paneth cells produce several kinds of AMPs, while all IECs secrete cytokines and chemokines [116]. Leukocytes are drawn to the intestine by the concurrent release of cytokines and chemokines, where they can serve as antigen-presenting cells and control lymphocyte responses within the intestine [117]. The lamina propria plays a particularly important role in the fight against infections, as it is the last layer between the contents of the intestinal lumen and the host [118]. There are several kinds of immune cells located there: around 90% of all GI lymphocytes, alongside with granulocytes, macrophages and dendritic cells.

However, not all antigens and toxins can be neutralized by the broad immune system of the intestines. The liver serves as the primary organ barrier for the gut-derived antigenic load and protects the systemic circulation against both residual oxidative and pathogen burden originated from the gastrointestinal tract. Its large resident immune cell population, along with the hepatocytes, produces a wide range of cytokines, chemokines, and acute-phase proteins. Inflammatory processes in this organ are highly regulated, as they serve an especially important role in the fight against infections, toxins, carcinogenic agents, and they also help to maintain immunotolerance to avoid chronic inflammatory diseases [119].

Besides its immunological importance, the liver also serves as the major metabolic organ, as it is responsible for several essential pathways. It metabolizes and synthetizes proteins, carbohydrates and fats, stores glycogen and various vitamins, removes toxins from the blood circulation, destroys red blood cells, participates in the hemoglobin metabolism, and secretes bile [120–122]. It has a right and a left lobe connected cranially at the midline, which have separate hepatic ducts entering the gall bladder. From the gall bladder, which is located on the visceral surface of the right lobe, two bile ducts reach the mid-distal part of the duodenum. The parenchyma of the chicken liver is quite similar to mammals, except it lacks lobules and intralobular trabeculae [123].

The blood arriving from the gut through the portal vein mixes with oxygen-rich blood from the hepatic artery, flows through the sinusoids, and leaves the liver through the central veins. There are many openings (fenestrations) between the endothelial cells that surround the sinusoids, allowing contact between substances of intestinal origin and hepatocytes and non-parenchymal cells [124]. First, the hepatocytes and the resident macrophages of the liver, the Kupffer cells recognize PAMPs and damage associated molecular patterns (DAMPs) with a wide range of pattern recognition receptors (PRRs) [125, 126]. As the liver tries to protect the body from excessive inflammation, these molecules induce phagocytosis and get degraded

by the cells without the release of inflammatory mediators [119]. The liver's resident immune cells fill the sinusoids and the subendothelial compartment, also known as the space of Disse, where lymph gathers and passes into the lymphatic arteries adjacent to the portal tract. These cells are mostly different kinds of APCs, myeloid-, and lymphoid cells [119]. Among myeloid cells, Kupffer cells are the most important as they take up almost the third of the non-parenchymal cells of the liver, while they are 90% of all fixed macrophage population in the body. They express several immune receptors (including TLR2, 3, 4, 5, 7, and 15 [78]), and have an important role in phagocytosis, cytokine production, tissue repair mechanisms, immune regulation and liver regeneration processes [127]. Additionally, a number of studies have demonstrated the presence of several AMPs in the liver, which are produced in response to infections and assist in immune regulation and the defense against infectious pathogens [128–130].

As can be seen above, the gut-liver axis is of particular importance in the management of GI infections. Broiler chickens with an immature immune system are prone to develop dysbacteriosis and necrotic enteritis, which leads to the disruption of the intestinal integrity and the leakage of microbial toxins and byproducts through the epithelial barrier. Consistent inflammation and its associated adverse effects result in repressed weight gain and thus decreased productivity in poultry [131]. Modeling these pathologies *in vitro* is relevant to finding potential molecules for the prophylaxis of intestinal and hepatic inflammatory and oxidative damage induced by gut-originated pathogen load.

3.5 Benefits and limitations of *in vitro* cell cultures and explant cultures

Cell cultures are an essential part of scientific research today, contributing significantly to reducing the number of animals used in experiments. They provide a cost- and time-efficient, easy way to test various pharmaceuticals or toxic substances, and they are also widely used in the field of various disease and cancer research. Although they have many advantages, cell cultures cannot be considered the best solution to every scientific question. However, if their limitations are recognized, they can be an extremely valuable tool to study a variety of ideas.

The first cell cultures were created by using small pieces of organs, where the cells migrated from the removed tissue into the surface. Ross Harrison (1870-1959) was the first to successfully grow tissue outside of an organism while studying the nerve development of frogs in 1907, and he also established the “hanging drop” technique [132]. Alexis Carrell (1873-1944) and Montrose Burrows (1884-1947) further advanced these methods, inventing new solutions for cell culture media and plate surfaces [133]. They successfully cultured tissues from

embryonic and adult tissues of chicken, dog, cat, rat, and guinea pig [134]. Carrell developed the first cell line, which was maintained from 1912 to 1946, two years after his death, by his assistant. Carrell worked together with Charles Lindbergh (1902-1974) and they contributed hugely to this area through the years [135].

However, cell cultures did not become popular until the early 1940s, when a synthetic cell culture media was designed, which allowed scientists to maintain cell lines for a prolonged time [136]. One of the firsts to achieve that was Wilton R. Earle (1902-1964), who managed to isolate the first continuous cell line from mouse fibroblasts in 1943 [137], and later he also developed the first cell line from colon cells [138]. In 1951, the first human cell line was obtained by George Otto Gey (1899-1970) from Henrietta Lacks, who suffered, and later died from cervical cancer. The HeLa cell line later contributed to several life-changing scientific discoveries, for example, the polio vaccine, and is still used by various research teams in the areas of virology, cancer, genetics, and toxicology [135]. Nowadays many cell lines are available on the market, mostly from cells isolated from cancerous or embryonic tissue.

Cell lines are undoubtedly useful, as they are cost-efficient and easy to maintain in a laboratory environment, besides, they consist of a uniform population of cells providing us with a more homogenous sample. On the other hand, continuous cell line passage can significantly alter their genetic, and consequently their phenotypic, physiological, and metabolic profile, which can eventually lead to subculture heterogeneity. Because of this, cell lines do not always accurately reflect conditions *in vivo* and might produce unexpected or, in rare situations, even deceptive results [139]. Another threatening factor is the contamination of cell lines with either intracellular pathogens (e.g. *Mycoplasma*) or other cell lines. Results from a wide-ranging screening investigation indicate that HeLa cells were present in the majority of cell lines that cell banks distributed and used around 1970-80 internationally [140]. In light of this, it's critical to be cautious when interpreting the results of cell line studies.

A better solution may be the use of primary cell cultures, which are cell fractions isolated from an organism and maintained only for a couple of days, not leaving enough time for genetic modifications. To isolate cells from tissues, at first, the extracellular matrix needs to be disturbed by proteolytic enzymes, such as trypsin and collagenase, and with agents that can bind the Ca^{2+} on which cell-cell adhesion depends, such as ethylene-glycol tetra-acetic acid (EGTA) [141]. The traditional method relies on cells being exposed to a surface coated with an extracellular matrix component, most commonly collagen, which is a key component of the extracellular matrix and therefore plays an important role in the formation of the natural medium, without which cells cannot differentiate [141]. It also helps them to surface and monolayer and increases their lifespan. Depending on the cell type, other extracellular matrix components such as fibronectin or laminin may be used, but also synthetic molecules such as poly-L-lysine. A single layer of cell culture is formed on the surface of a properly coated culture

dish, in which cells can grow at the same rate, as they can take up similar amounts of nutrients. Many researchers still use this method today because it is simple and effective, but the disadvantage is that the cell cultures created by this method are far from the natural structure of living organelle tissues. In some ways, sandwich culture has proven to be more favorable than the traditional technique for the cultivation of many cells. It involves placing the cells between two layers of extracellular matrix, polyacrylamide or collagen. In sandwich culture, the shape and function of the cells more closely resemble that of living tissue [142].

There is also the possibility to culture more than one type of cell at the same time. These are called co-cultures, and, in contrast with mono-cultures, can be used to identify the interactions between different cell types [143]. In co-culture models, three forms of cell-cell interactions are apparent: cell adhesion, cell-extracellular matrix connection, and paracrine communication via soluble chemicals. Depending on the culture environment, these three forms of interactions can exist separately or have an impact on the cell cultures simultaneously. Cells in co-cultures can either be mixed with each other prior to seeding, or they can be separated by a porous membrane, depending on the intended use and on the cell types [144].

Another aspect of studying tissues are the *ex vivo* explant cultures. They are small pieces of tissue isolated from an alive or euthanized animal, or from humans through surgery or biopsy. Although their lifespan is shorter, they have characteristics much closer to the *in vivo* conditions [145]. For example, in the intestine, it would be especially complicated to study the complex interface epithelial cells have with a variety of immune cells using cell culture techniques, therefore, explants are considered more favorable in this case [146]. The first ones to isolate explants from human small intestine were Browning and Tier in 1969, who, with this discovery, opened the path to use intestinal explant cultures for a variety of goals – e.g. to study the gut homeostasis, the pathogenesis of gut-related diseases, enzyme and hormone production, cancer development, or the effect of drugs and toxins in the intestine [147]. The main limitation of explant cultures is their sensitivity, as they require an oxygen-rich environment and a liquid-tissue-gas interface to avoid cell necrosis [148]. One possible solution would be to decrease the size of the explants, and to use younger donors, as their cells could be more resistant to anoxia [149]. The method of Zhan et al. [150], which was further advanced by our research group [145] allows us to isolate tissue samples in an easy and quick way using a punch biopsy sampler, and to maintain them without the presence of tissue necrosis for the duration of the treatments by choosing the right size.

3.5.1 Inflammatory Cell Culture Models

A significant area of cell culture use is the investigation of different inflammatory and immunological pathways and mechanisms. In order to study an anti-inflammatory or immunomodulatory molecule, an effective way to induce inflammation in the cell culture is

inevitable. The agent used to activate inflammatory pathways must be chosen carefully, considering the cell types used in the study, the mechanism of action of the molecules to be tested and the potential cytotoxicity.

PAMPs are one of the most commonly used substances for these purposes. They are derived from pathogenic bacteria and viruses that trigger inflammatory responses when recognized by the PRRs of the immune cells. Many PAMPs have been described and used both in *in vivo* and *in vitro* studies, the most common ones are proteins, lipoproteins, RNA and DNA species [151].

In order to investigate the effect of immunomodulatory substances, there is a need for an effective inflammatory model. Several types of PAMPs may be applied to trigger cellular inflammatory and stress response in *in vitro* cultures. The TLR agonists of the highest importance and clinical relevance are LPS derived from the Gram-negative [152], and LTA derived from the Gram-positive bacterial cell wall [153]. LPS and LTA can both bind nonspecifically to membrane phospholipids of red blood cells, however, in most cell types they bind to TLR4 (in the case of LPS), TLR2 (in the case of LTA) or to CD14 receptors and activate the NF-κB pathway, which leads to the production of several pro-inflammatory cytokines, depending on the cell type [154]. LPS from O55:B5 chicken pathogen *Escherichia coli* is most frequently used *in vitro* on chicken cell lines and cultures [155, 156]. *Staphylococcus aureus* LTA proved to induce oxidative burst via TLR activation initiated Protein kinase C (PKC) dependent transduction in chicken heterophil granulocytes [157].

Phorbol myristate acetate (PMA) is commonly used in human medical research both *in vivo* and *in vitro* to induce inflammation and thus challenge the therapeutic effects of substances with anti-inflammatory nature. Applying PMA was coupled to elevated proinflammatory cytokine release and COX-2 expression [158, 159]. Meanwhile, the oxidative response induced by PMA is remarkably stronger than the ones triggered by LTA and LPS stimulation; a selective PKC inhibitor could block this activation. Therefore, it presumably activates the cellular inflammatory response in a somewhat different manner [160].

4 Significance and aims of the study

The necessity for developing antibiotic substitutes continues to increase due to the threat posed by antimicrobial resistance. The replacement of antibiotic therapy is especially crucial for chickens, as they are representing the majority of farm animals. Even in subclinical forms, infections can impair productivity by weakening the flock. Infections of intestinal origin are among the most common in poultry, therefore the investigation of the intestinal and hepatic immune response is of great importance. AMPs could provide a feasible solution for the treatment of infectious diseases among poultry, however, several questions arise before they can enter clinical trials. This PhD thesis tries to answer some of these questions.

Our research group has already established and characterized a primary co-culture comprised of hepatocytes and non-parenchymal cells (predominantly resident macrophages, the Kupffer cells) and an intestinal explant model, both of which can serve as a potent tool to investigate inflammatory processes related to infections in chickens. In order to do that, first, an inflammatory model had to be established, therefore this was our first goal. As former studies presented that this kind of cell culture model reacts somewhat differently to inflammatory stimuli when compared to cell cultures of mammalian origin, several PAMPs were needed to be tested.

Our next question was whether the AMPs chosen for this study, Cath-2 and IDR-1002 have any decreasing effect on the viability of cells in either the hepatocyte – non-parenchymal cell co-culture or the intestinal explants. As AMPs have been proven to have damaging effects on cell membranes and even found to be toxic to some cell types when applied in higher concentrations, it is certainly an important aspect to determine for their usability.

Our main goal, besides investiganting their potential toxicity was to define the immunomodulatory effects these peptide may have on our models. As most AMPs, Cath-2 and IDR-1002 are also proven to have broad immunomodulatory effects, and they affected the immune function of different cell types diversly. Confirmed by previous studies that these effects can vary between species and cell types, we believe that it is cruicial to have reliable information on the specific characteristics these peptides have on the hepatic and intestinal cells of chickens. Furthermore, the recognition of the interrelations between redox and immunological processes in our models may be of great importance.

Goals of my PhD thesis are summarized as follows:

Ad 1, to develop an inflammatory model on our hepatocyte – non-parenchymal cell co-culture model of chicken origin.

Ad 2, to test the effect Cath-2 and IDR-1002 have on the cell viability of the hepatocyte – non-parenchymal cell co-culture and the intestinal explants.

Ad 3, to investigate the immunomodulatory effects of Cath- 2 on the hepatocyte – non-parenchymal cell co-culture and the intestinal explants.

Ad 4, to investigate the immunomodulatory effects of IDR-1002 on the hepatocyte – non-parenchymal cell co-culture and the intestinal explants.

5 Materials and methods

5.1 Ethic statement

The hepatocytes and non-parenchymal cells for the hepatic cell cultures and the intestinal explants were isolated from a 3-week-old male Ross-308 broiler chicken. All experiments were in accordance with European Union laws, institutional guidelines, confirmed by the Local Animal Welfare Committee of the University of Veterinary Medicine Budapest, and permitted by the Government Office (number of permission: GK-419/2020; approval date: 11 May 2020).

5.2 Chemicals and reagents

Unless stated otherwise, all chemicals were purchased from Merck (Darmstadt, Germany).

5.3 Study design

The present work aimed to investigate the immunomodulatory effects of two AMPs on the immune response of chicken, Cath-2 and IDR-1002. Both *in vitro* models employed in the studies were developed by our research group: first, the peptides were studied on a hepatocyte – non-parenchymal primary cell co-culture and then an intestinal explant model was used.

Five different experiments (Preliminary study and Studies I-IV) have been conducted as part of the present dissertation. The preliminary study aimed to establish a reliable and reproducible inflammatory model on the hepatic cell culture developed earlier by our research group. In Study I and Study II, the immunomodulatory effects of the two AMPs, Cath-2 and IDR-1002 were investigated on the hepatocyte – non-parenchymal cell co-cultures of chicken origin. Then, in Study III and Study IV, the same peptides were applied to chicken intestinal explant cultures to observe their effect on the immune response of the gut wall. The development of the intestinal explant isolation and culturing methodology and the assessment of various pro-inflammatory agents in the explant cultures were also performed by our research group, but as these studies are not part of this dissertation, they will not be discussed in such detail as in the case of the hepatic cell cultures.

All studies include the measurement of cell viability parameters, as it is inevitably important to investigate the potential toxic effects of the compounds. First of all, it is crucial to recognize whether the results are affected by excessive damage of the cells, for example,

reduced cytokine production can be caused by a reduction in cell number or diminished cell viability. Second, the potential future *in vivo* use of AMPs also necessitates knowing the correlation between different concentrations and possible harmful effects on cell viability. To monitor this, two types of tests were employed. Metabolic activity was measured by the CCK (cell counting kit) test and extracellular lactate dehydrogenase (LDH) activity was assessed to show damage to the cell membrane, as this intracellular enzyme only escapes the cells if membrane is not intact. These two parameters together provide sufficient information on cell viability levels.

Several inflammatory and redox parameters were also measured during the studies. PMA and LTA as pro-inflammatory agents were chosen based on the data gained from the Preliminary study. As the results from Study I showed no promising effects regarding PMA, it was decided to leave it out from Study II. PMA was also applied to the intestinal explants in a study which is not part of this dissertation, but as it had almost no pro-inflammatory effects on the explants, it was not included in Study III and Study IV.

The study design is explained in more detail in **Table 1**.

Table 1.: Study design

	Model	Investigated compounds	Cell viability parameters	Inflammatory parameters	Redox parameters
Preliminary Study	Chicken hepatocyte – non-parenchymal cell co-culture	LPS, LTA, PMA	Metabolic activity and extracellular LDH activity	CXCLi2 and IL-6 concentrations	-
Study I	Chicken hepatocyte – non-parenchymal cell co-culture	LTA, PMA Chicken cathelicidin-2	Metabolic activity and extracellular LDH activity	CXCLi2, IFN-γ, IL-10 and M-CSF concentrations	H_2O_2 and MDA levels
Study II	Chicken hepatocyte – non-parenchymal cell co-culture	LTA and IDR-1002	Metabolic activity and extracellular LDH activity	CXCLi2, IL-6, IL-16, IFN-γ, IL-10, M-CSF and RANTES concentrations	H_2O_2, Nrf2 and PC levels
Study III	Chicken intestinal explant	LTA and Chicken cathelicidin-2	Metabolic activity and extracellular LDH activity	CXCLi2, IL-6, IL-2, IFN-γ, IL-10 concentrations and IFN-γ/IL-10 ratio	-
Study IV	Chicken intestinal explant	LTA and IDR-1002	Metabolic activity and extracellular LDH activity	CXCLi2, IL-2, RANTES, IFN-γ, IL-10 concentrations and IFN-γ/IL-10 ratio	-

5.4 Preliminary study: induction of inflammation

5.4.1 Cell isolation

The hepatocytes and non-parenchymal cells were isolated from a 3-week-old male Ross-308 broiler chicken. The animal arrived to our laboratory from the hatchery of Gallus Kft. (Devecser, Hungary), was fed according to Ross Technology, feed and water were offered ad libitum and all efforts were made to support the welfare of the animal.

Slaughter of the chicken was done by decapitation. After opening the body cavity and accessing the liver, the portal system was cannulated through the *gastropancreaticoduodenal* vein (**Figure 4**). In order to remove the blood and disrupt the connective tissue of the organ, a three-step perfusion was performed with 150 mL of 0.5 M EGTA containing Hanks' Balanced Salt Solution (HBSS) buffer, 150 mL EGTA-free HBSS and in the final step, 100 mL HBSS supplemented with 100 mg collagenase type IV, 7 mM $CaCl_2$ and 7 mM $MgCl_2$ (Nordmark, Uetersen, Germany) as described previously by Mackei et al [161]. After removal of the liver and the disruption of the Glisson's capsule, the cells were suspended in 50 mL ice cold bovine serum albumin (BSA, 1.25 g) containing HBSS buffer and filtered through three layers of sterile gauze. To prevent cell aggregation, this cell suspension was then kept on ice for 45 minutes. Following this, the suspension was centrifuged three times at 100x g for 3 minutes. The pellet containing hepatocytes was resuspended in Williams Medium E supplemented with 0.22% $NaHCO_3$, 50 mg/mL gentamycin, 0.5 µg/mL amphotericin B, 2 mM glutamine, 4 µg/L dexamethasone, 20 IU/L insulin, and 5% fetal bovine serum (FBS). The FBS was only present in the medium for the first 24 hours after seeding.

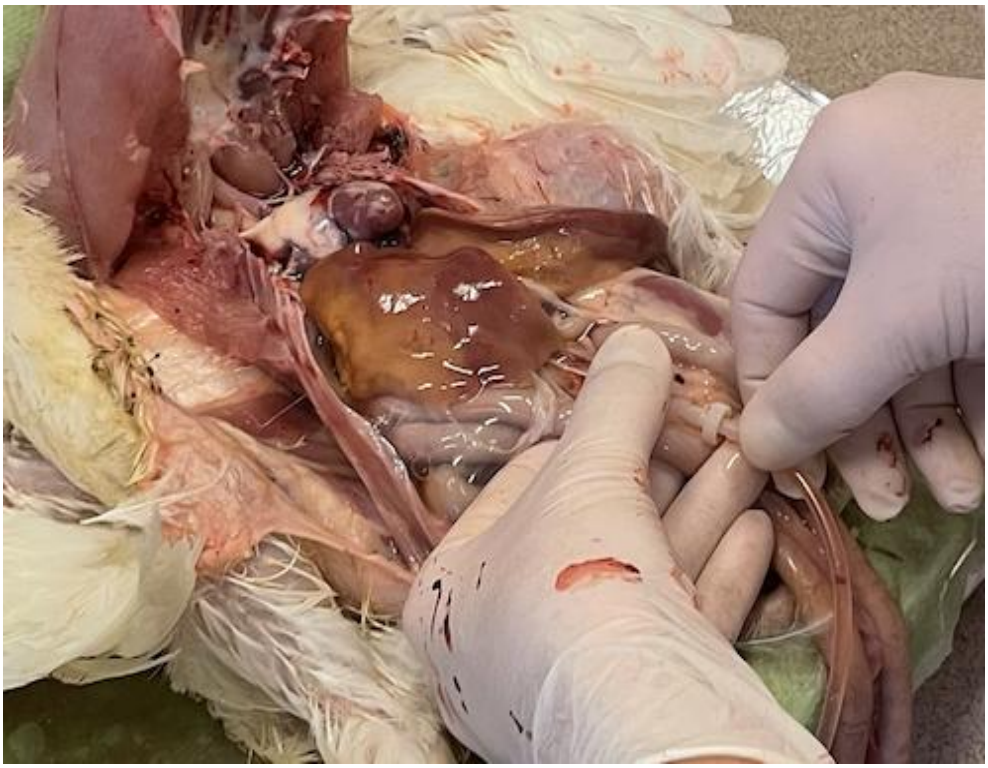

Figure 4. Perfusion of the chicken liver with collagenase-containing buffer solution.

To guarantee that any remaining hepatocytes, cell debris, and erythrocytes were sedimented, the supernatant containing non-parenchymal cells was centrifuged once more at 350x g for 10 minutes. Thereafter, the supernatant was centrifuged at 800x g for 10 minutes, and the pellet was resuspended in Williams Medium E. Viability of the isolated hepatocytes and non-parenchymal cells was confirmed by trypan blue exclusion test. The cell suspensions were diluted to 8.5*105 cells/mL in case of the hepatocyte-enriched fraction and to $1.5*10^5$ cells/mL in case of the non-parenchymal cell-containing fraction. Both hepatocyte and non-parenchymal cell-enriched fractions (the latter mostly containing macrophages) have been previously characterized by flow cytometry and immunofluorescent detection of specific markers for hepatocytes and macrophages [161].

After mixing the cell suspensions in the ratio of 6:1 (hepatocytes to non-parenchymal cells) – which modeles a mild hepatic inflammation and therefore creates the conditions for an inflammatory response – the hepatocyte- non-parenchymal cell co-cultures were seeded onto 96-well plates (Greiner Bio-One Hungary Kft., Mosonmagyaróvár, Hungary) previously coated with collagen type I (10 μg/cm^2). The seeding volume was 100 μL/well. The cell cultures were incubated at 37°C in humid atmosphere with 5% CO_2. Culture media were changed after 4 hours and confluent co-cultures were gained after 24 hours (**Figure 5**). Culture medium contained 5% FBS only in the first 24 hours of culturing. Other supplements added to the medium during the experiment were the same as in the medium used for the seeding.

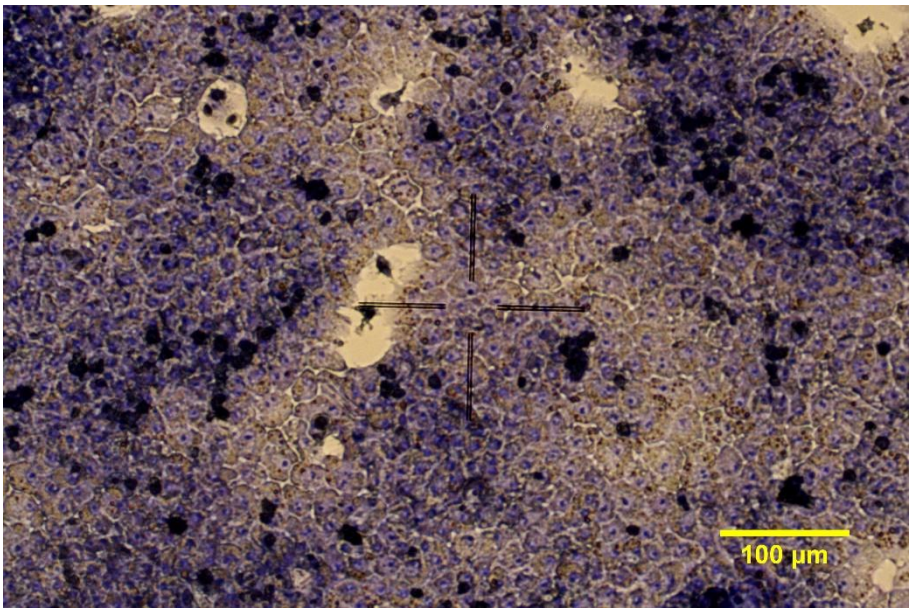

Figure 5. Giemsa staining of chicken hepatocyte – non-parenchymal cell co-cultures 24 hours after seeding (bar line = 100 µm)

After conducting the study involving LPS and LTA, our research group have decided to test several other PAMPs for the induction of inflammation. Due to the length and the scope of this present PhD thesis, it was decided to only include the results after PMA treatment. The rest of the study can be found and is freely accessible in the relevant article (Sebők et al., 2021 – can be found under "Own scientific publications"). Cells for the two parts of the study (LPS/LTA and PMA) were isolated from two different chickens at two different times, that is why two Control groups are used and the results are displayed in two sets of diagrams.

5.4.2 Treatment of cell cultures

In the first part of the study, the medium of the cell cultures was supplemented with 0 (control), 10 or 50 µg/mL LPS from *Escherichia coli* (O55:B5), and with 10 or 50 µg/mL LTA from *Staphylococcus aureus* (referred to as LPS/LTA study onwards, $n_{(LPS/LTA)}$=6/group). In the second part of the study, cell culture medium was supplemented with 0 (control), 100 or 1000 ng/mL PMA (referred to as PMA study onwards, $n_{(PMA)}$=5/group, except for metabolic activity, where $n_{(PMA)}$=10/group). Cells were incubated with the treatment solutions for 24 hours.

5.4.3 Measurements

5.4.3.1 Metabolic activity

The metabolic activity of the cells was measured on 96-well plates by CCK-8 assay (Cell counting Kit-8, Dojindo Molecular Technologies, Rockville, USA), detecting the amount of $NADH+H^+$ and $NADPH+H^+$produced in the catabolic pathways. The test was performed according to the manufacturer's protocol. First, 10 µL CCK-8 reagent and 100 µL fresh

Williams' Medium E were added to the cultured cells, and after a 2-hour incubation, the absorbance was measured at 450 nm with a Multiskan GO 3.2 reader (Thermo Fisher Scientific, Waltham, MA, USA).

5.4.3.2 Extracellular LDH activity

Extracellular lactate dehydrogenase (LDH) activity was measured from the cell culture media using an enzyme kinetic photometric assay (Diagnosticum Ltd., Budapest, Hungary). The method is based on the measurement of the NAD^+ formed via the enzymic reaction of LDH with pyruvate and $NADH+H^+$. Each well of a microplate was filled with 200 µL of the working reagent (56 mM phosphate buffer, pH = 7.5, 1.6 mM pyruvate, 240 M $NADH+H^+$) and 10 µL of sample. A Multiskan GO 3.2 reader was used to detect the absorbance at 340 nm. The LDH activity was estimated by measuring the absorbance six times in one-minute increments while incubating the mixture at 37°C and averaging the changes between the consecutive time points ($n_{(LPS/LTA)}$=6, $n_{(PMA)}$=5).

5.4.3.3 Cytokine concentrations

The concentrations of interleukin-6 (IL-6) and chicken chemotactic and angiogenic factor (CXCLi2, also known as chicken interleukin-8 [IL-8]) were measured in the culture media by chicken specific ELISA kits (MyBioSource, San Diego, CA, USA) following the manufacturer's protocol. The absorbance was measured at 450 nm with a Multiskan GO 3.2 reader ($n_{(LPS/LTA)}$=6, $n_{(PMA)}$=5).

5.4.4 Statistical analysis

All statistical analysis was performed in R v. 4.0.3 (R Core Team, 2020). Statistical significance was evaluated for each treatment to the corresponding control group using Wilcoxon signed rank test. If the p-value was less than 0.05 we have considered a difference significant. For visualization, values were converted to relative percentages, where the mean of the corresponding Control group was considered as 100%. Graphs were generated using GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA).

5.5 Study I and II: Effects of Cath-2 and IDR-1002 on a primary hepatic cell culture

5.5.1 Cell isolation

In both Study I and Study II, the preparation of hepatocyte – non-parenchymal cell co-cultures was carried out following the methodology explained in the Preliminary study. After isolation of the cells from a 3-week-old Ross-308 broiler chicken, cell cultures were prepared

using 24-well and 96-well culture plates (Greiner Bio-One, Frickenhausen, Germany) pre-coated with collagen type I. On the 24-well plates, the seeding quantity was 400 µL/well, while 100 µL/well on the 96-well plates.

5.5.2 Treatment of cell cultures: Study I

After the 24-hour incubation time of seeded cell cultures, the media was removed and replaced by culture media containing the investigated agents. Cells were treated with Cath-2 in 5 nmol/mL and 10 nmol/mL concentrations (Isca Biochemicals, Exeter, Devon, UK). *Staphylococcus aureus*-derived LTA (Sigma-Aldrich, Darmstadt, Germany) was used in 50 µg/ml, and PMA (Sigma-Aldrich, Darmstadt, Germany) in 1000 ng/mL concentrations. The Control group received only Williams Medium E, without any supplementation. The treatment groups are presented in **Table 2** (n=6/group). After another 24 hours of incubation, samples were taken from the cell culture media and stored at -80°C until further measurements. 96-well plates were used for the measurement of the metabolic activity of the cells.

Table 2. Treatment groups in Study I

Treatment group	Cathelicidin-2	LTA	PMA
Control	-	-	-
Cath-low	5 nmol/mL	-	-
Cath-medium	10 nmol/mL	-	-
LTA	-	50 µg/mL	-
LTA+Cath-low	5 nmol/mL	50 µg/mL	-
PMA	-	-	1000 ng/mL
PMA+Cath-low	5 nmol/mL	-	1000 ng/mL

5.5.3 Treatment of cell cultures: Study II

After 24 h culturing, cells were exposed to the following substances for 24 h. IDR-1002 (Isca Biochemicals, Exeter, Devon, UK) was added in concentrations of 10, 30, and 90 µg/mL (IDR-low, IDR-medium, and IDR-high, respectively) alone and in combination with 50 µg/mL LTA from *Staphylococcus aureus*. The Control group received only Williams Medium E, without any supplementation. The treatment groups are presented in Table 2 (n=6/group). After sampling from the cell culture media of 24-well plates, cells were lysed by applying 50 µL/well Mammalian Protein Extraction Reagent (M-PER) lysis buffer and scraped from the surface. Culture media and cell lysate samples were stored at -80°C until further processing. 96-well plates were used for the measurement of the metabolic activity of the cells. Treatment groups are explained in **Table 3.**

Table 3. Treatment groups in Study II

Treatment group	IDR-1002	LTA
Control	-	-
IDR-low	10 μg/mL	-
IDR-medium	30 μg/mL	-
IDR-high	90 μg/mL	-
LTA	-	50 μg/mL
LTA + IDR-low	10 μg/mL	50 μg/mL
LTA + IDR-medium	30 μg/mL	50 μg/mL
LTA + IDR-high	90 μg/mL	50 μg/mL

5.5.4 Measurements

5.5.4.1 Metabolic activity

This test was carried out following the same protocol described in the Preliminary study, **for both Study I and Study II**

5.5.4.2 Extracellular lactate dehydrogenase (LDH) activity

This test was carried out following the same protocol described in the Preliminary study, **for both Study I and Study II**

5.5.4.3 Cytokine concentrations

Chicken specific ELISA kits (MyBioSource, San Diego, CA, USA) were employed following the manufacturer's instructions to measure the following cytokines and chemokines. Absorbance values were obtained with a Multiskan GO 3.2 reader at 450 nm.

- **Study I:** CXCLi2 concentrations from the cell culture media of the 24-well plates.
- **Study II:** CXCLi2 and IL-6 from the cell culture media of the 24-well plates.

Luminex xMAP technology was used to determine the following protein concentrations, performing Milliplex Chicken Cytokine/Chemokine Panel (Cat.Nr.: GCYT1-16K, Merck KGaA, Darmstadt, Germany) according to the instructions of the manufacturer.

- **Study I:** IFN-γ, IL-10, and macrophage colony-stimulating factor (M-CSF) concentrations were measured.
- **Study II:** IL-16, IFN-γ, IL-10, M-CSF and RANTES (Regulated And Normal T-cell Expressed and Secreted) were measured.

Briefly, all samples were thawed and tested in a blind-fashion and in duplicate. 25 ml volume of each sample, standard, control, and assay buffer was added to a 96-well plate (provided with the kit). An additional 25 μl of the distinctly colored, capture antibody-coated

bead sets were added to each well. After overnight incubation, biotinylated detection antibody mixture and streptavidin phycoerythrin were added to the plate following appropriate washing steps. After the last washing step, 150 ml drive fluid was added to the wells, the beads were resuspended for an additional 5 minutes on a plate shaker and read on the Luminex MAGPIX® instrument. Luminex xPonent 4.2 software was used for data acquisition. Five-PL regression curves were generated to plot the standard curves for all analytes by the Milliplex Analyst 5.1 (Merck Millipore, Darmstadt, Germany) software calculating with bead median fluorescence intensity (MFI) values.

5.5.4.4 Redox parameters

In both Study I and Study II, the fluorometric Amplex Red method (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect **extracellular H_2O_2 content** in the culture medium. A Victor X2 2030 fluorometer (Perkin Elmer, Waltham, MA, USA) was used to detect fluorescence (λex = 560 nm, λem = 590 nm) after a 30-minute incubation of 50 µL freshly prepared Amplex Red (100 µM) and HRP (0.2 U/mL) containing working solution with 50 µL culture medium at room temperature (21°C).

In study I, a specific colorimetric assay was used to detect **MDA concentration** as a marker of lipid peroxidation in cell culture medium. 300 µL of freshly prepared thiobarbituric acid stock solution was mixed with 100 µL cell culture medium according to the protocol. After 1 hour of incubation at 95°C, the solutions were cooled on ice for 10 minutes. A Multiskan GO 3.2 reader was used to measure absorbance at 532 nm.

In Study II, protein carbonyl (PC) levels were measured by a chicken specific ELISA kit (MyBioSource, San Diego, CA, USA) from the cell lysate samples of the 24-well plates.

5.5.4.5 Nrf2 concentrations

In Study II, nuclear factor erythroid 2-related factor 2 (Nrf2) was measured in the culture media of the 24-well plates using a chicken specific ELISA kit (MyBioSource, San Diego, CA, USA).

5.5.4.6 Total protein concentration

In Study II, total protein concentrations were measured from the cell lysate samples with the Pierce™ Bicinchoninic Acid (BCA) Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA) as indicated by the manufacturer, with BSA as standard. With this method, the reduction of Cu^{2+} to Cu^{1+} can be measured, as BCA makes a water soluble, purple-colored complex with Cu^{1+}. 25 µL of sample was added to 200 µL of reagent mixture, and absorbance was measured after 30 min incubation at 37°C at 562 nm with a Multiskan GO 3.2 reader. The values were used to standardize the PC values which were measured from the same cell lysate samples.

5.5.5 Statistical analysis

All statistical analyses were performed in R v. 4.0.3 (R Core Team, 2020). Pairwise comparisons were performed using Wilcoxon signed-rank test, as some of the treatment groups showed non-normal distribution based on Shapiro-Wilk tests. We have considered a difference statistically significant if the p-value was less than 0.05.

In Study I., Cath-low, Cath-medium, LTA and PMA groups were compared with the Control, LTA+Cath-low with the LTA group and PMA+Cath-low with the PMA group.

In Study II., IDR-low, IDR-medium, IDR-high and LTA groups were compared with the Control, and LTA+IDR-low, LTA+IDR-medium and LTA+IDR-high with the LTA group.

For visualization, the metabolic activity and the LDH activity values were converted to relative percentages, where the mean of the Control group was considered as 100%. Graphs were generated using GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA).

Scatterplots between the measured parameters for each treatment groups in Study I were created using R v. 4.0.3 (R Core Team, 2020). These plots were supplemented with linear regression lines (along with a 95% confidence bands) and Spearman correlation coefficients with p-values to further visualize the association of the parameters.

5.6 Study III and IV: Effects of Cath-2 and IDR-1002 on an intestinal explant culture

5.6.1 Preparation of the explants

The isolation of the intestinal explants was performed using the methodology recently developed by our research team [162], for which a 3-week-old male Ross-308 broiler chicken was sacrificed.

The chicken was decapitated, and after opening the coelomic cavity aseptically in a dorsal position, the small intestines were removed. In a 10 cm distance from the Meckel's diverticulum, a 13 cm long ileal segment was excised followed by the removal of adipose tissue from its outer surface (see **Figure 6**). Furthermore, both the outer and the inner side (with a sterile feeding needle) of this section were washed multiple times by using phosphate-buffered saline (PBS) + 1% penicillin-streptomycin solution (Pen-Strep, Gibco [Waltham, MA USA]). Thereafter the intestine was cut longitudinally and washed with the same solution until the lack of visible contamination. Following this, the intestinal section was placed in ice-cold, sterile PBS + Pen-Strep solution and the excision of the explants was performed in a sterile environment.

Figure 6. Preparation of the intestinal explants

The segment was placed on an ice-cold, sterile glass plate with the mucosal side upwards, and was fixed with glass slides. During the process, the surface of the segment was rinsed periodically with Pen-Strep + PBS solution to keep the tissue moist. The removal of the explants was performed using biopsy punches (MDE GmbH [Heidelberg, Germany]) with a diameter of 1.5 mm. The explants were transferred to a 96-well cell culture plate pre-coated with type I collagen and filled with cell culture medium. The medium used for the experiment was Dulbecco's Minimal Essential Medium-F12 supplemented with 2.5 % FBS, 1% glutamine, 1% Pen-Strep, and one dose of HCM™ SingleQuots™ Kit (Lonza-Biocenter [Szeged, Hungary]). The latter contains ascorbic acid, BSA, hydrocortisone, transferrin, human Epidermal Growth Factor (hEGF), insulin, gentamicin, and amphotericin-B. The gained explants were incubated at 37°C and 5% CO_2 for 2 hours. **Figure 7.** shows a microscopic image of an explant after 14 hours of incubation, without any treatment, confirming that the intact micromorphology of the isolated explants was maintained during the entire experiment.

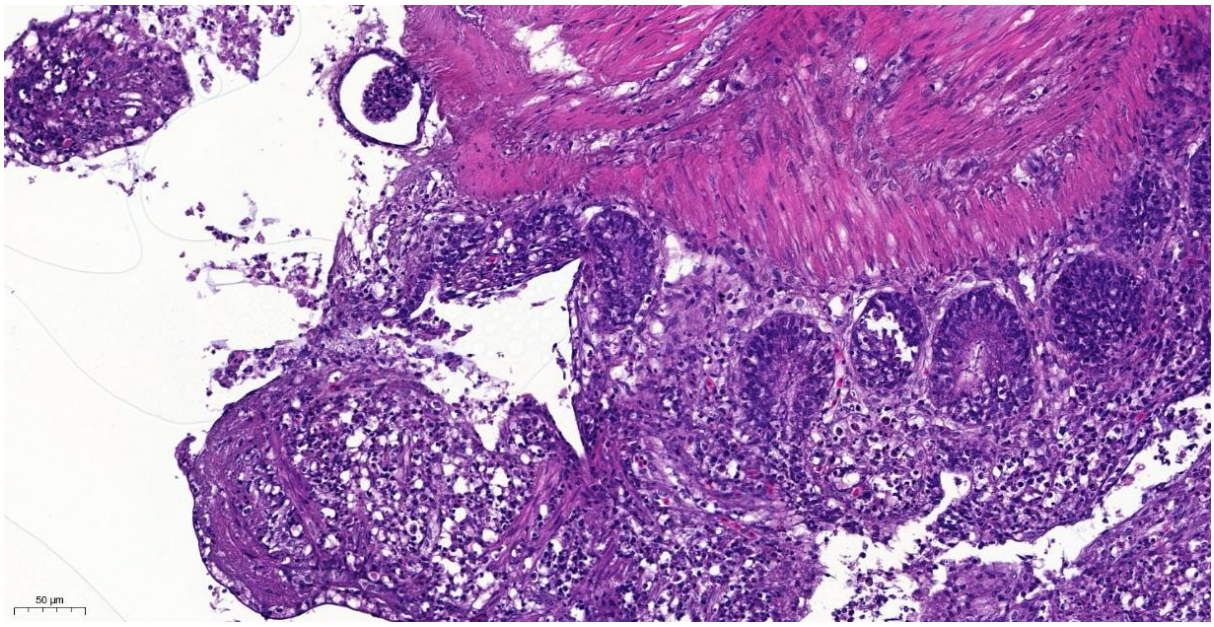

Figure 7. Microscopic image of a hematoxylin and eosin (H&E) stained section of an intestinal explant after 14 hours of incubation without treatment. Bar line=50 µm

5.6.2 Treatment of the explants: Study III

After the 2-hour incubation, cell culture medium was removed from the wells and fresh medium substituted with the treatment solutions was added. The treatment solutions included 5, 10 and 25 nmol/mL chicken cathelicidin-2 (Cath-low, Cath-medium and Cath-high, respectively), 10 µg/mL *Staphylococcus aureus* derived LTA (LTA), and the combination of the formers (LTA+Cath-low, LTA+Cath-medium and LTA+Cath-high). Control group received only fresh cell culture medium. Explants were incubated with the solutions for 12 hours, thereafter samples were taken from the cell culture medium and stored at -80°C until further processing. Treatment groups are explained in **Table 4**.

Table 4. Treatment groups in Study III.

Treatment group	Cath-2	LTA
Control	-	-
Cath-low	5 nmol/mL	-
Cath-medium	10 nmol/mL	-
Cath-high	25 nmol/mL	-
LTA	-	10 µg/mL
LTA + Cath-low	5 nmol/mL	10 µg/mL
LTA + Cath-medium	10 nmol/mL	10 µg/mL
LTA + Cath-high	25 nmol/mL	10 µg/mL

5.6.3 Treatment of the explants: Study IV.

After the incubation, the medium was removed from the wells and replaced with treatment solutions using the medium as a solvent. The explants were treated with 10, 30, and 90 µg/mL concentrations of IDR-1002 (IDR-low, IDR-medium and IDR-high, respectively), 10 µg/mL LTA derived from *Staphylococcus aureus* (LTA), and the combinations of them (LTA+IDR-low, LTA+IDR-medium, LTA+IDR-high). Each group contained 6 explants, which were incubated for 12 hours with the treatment solutions in the same conditions as before. Treatment groups are explained in **Table 5.**

Table 5. Treatment groups in Study IV.

Treatment group	**IDR-1002**	**LTA**
Control	-	-
IDR-low	10 µg/mL	-
IDR-medium	30 µg/mL	-
IDR-high	90 µg/mL	-
LTA	-	10 µg/mL
LTA + IDR-low	10 µg/mL	10 µg/mL
LTA + IDR-medium	30 µg/mL	10 µg/mL
LTA + IDR-high	90 µg/mL	10 µg/mL

5.6.4 Measurements

5.6.4.1 Metabolic activity

This test was carried out following the same protocol described in the Preliminary study, **for both Study III and Study IV.**

5.6.4.2 Extracellular LDH activity

For both Study III and Study IV, a colorimetric LDH assay kit (Sigma-Aldrich, St. Louis, MO, USA) was used for the quantification of extracellular LDH activity as its elevation is indicative of cell membrane damage. Following the manufacturer's instructions, components were mixed at room temperature, and a standard dilution series was prepared. A 2 µL sample was measured into a clean 96-well plate and adjusted to 50 µL with the provided buffer solution. The change in absorbance was measured at 450 nm using the Multiskan GO 3.2 reader every minute until the levels of the most concentrated standards were reached. Enzyme activity was computed via the calibration curve described in the protocol.

5.6.4.3 Cytokine concentrations

In both **Study III and Study IV**, the concentration of CXCLi2 (chicken IL-8) was determined through a chicken-specific sandwich ELISA per the instructions provided by the manufacturer (MyBioSource, San Diego, CA, USA).

Luminex xMAP method (previously described at Study I-II) was used to determine the concentrations of the following cytokines and chemokines:

- **Study III:** IL-2, IL-6, IFN-γ and IL-10
- **Study IV:** IL-2, IFN-γ, IL-10 and RANTES

The measurement was performed following the manufacturers' instructions, as described previously.

In both Study III and Study IV, for a more precise assessment of the inflammatory state of the explants, the IFN-γ to IL-10 ratio was calculated.

5.6.5 Statistical analysis

For the statistical analysis, R version 4.0.3 software was used. Wilcoxon's signed rank tests were employed for pairwise comparisons as the data showed non-normal distribution using Shapiro-Wilk tests. A significance threshold of $p<0.05$ was used.

In Study III, Cath-low, Cath-medium, Cath-high and LTA groups were compared with the Control, and LTA+Cath-low, LTA+Cath-medium and LTA-Cath-high with the LTA group.

In Study IV, IDR-low, IDR-medium, IDR-high and LTA groups were compared with the Control, and LTA+IDR-low, LTA+IDR-medium and LTA+IDR-high with the LTA group.

Metabolic activity and LDH activity results were graphically represented as percentages, with the mean of the control group set at 100%. Graphs were generated using GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA).

6 Results

6.1 Preliminary study: induction of inflammation

No alteration was found in metabolic activity after LPS or LTA exposure (Figure 8/A), however, there was a significant decrease after applying 100 and 1000 ng/mL PMA treatment (p=0.004, p=0.004, respectively) (Figure 8/B).

Concerning the extracellular LDH activity significant increase was found after both (10 and 50 µg/mL) LTA concentrations (p=0.026, p=0.004, respectively) (Figure 8/C).

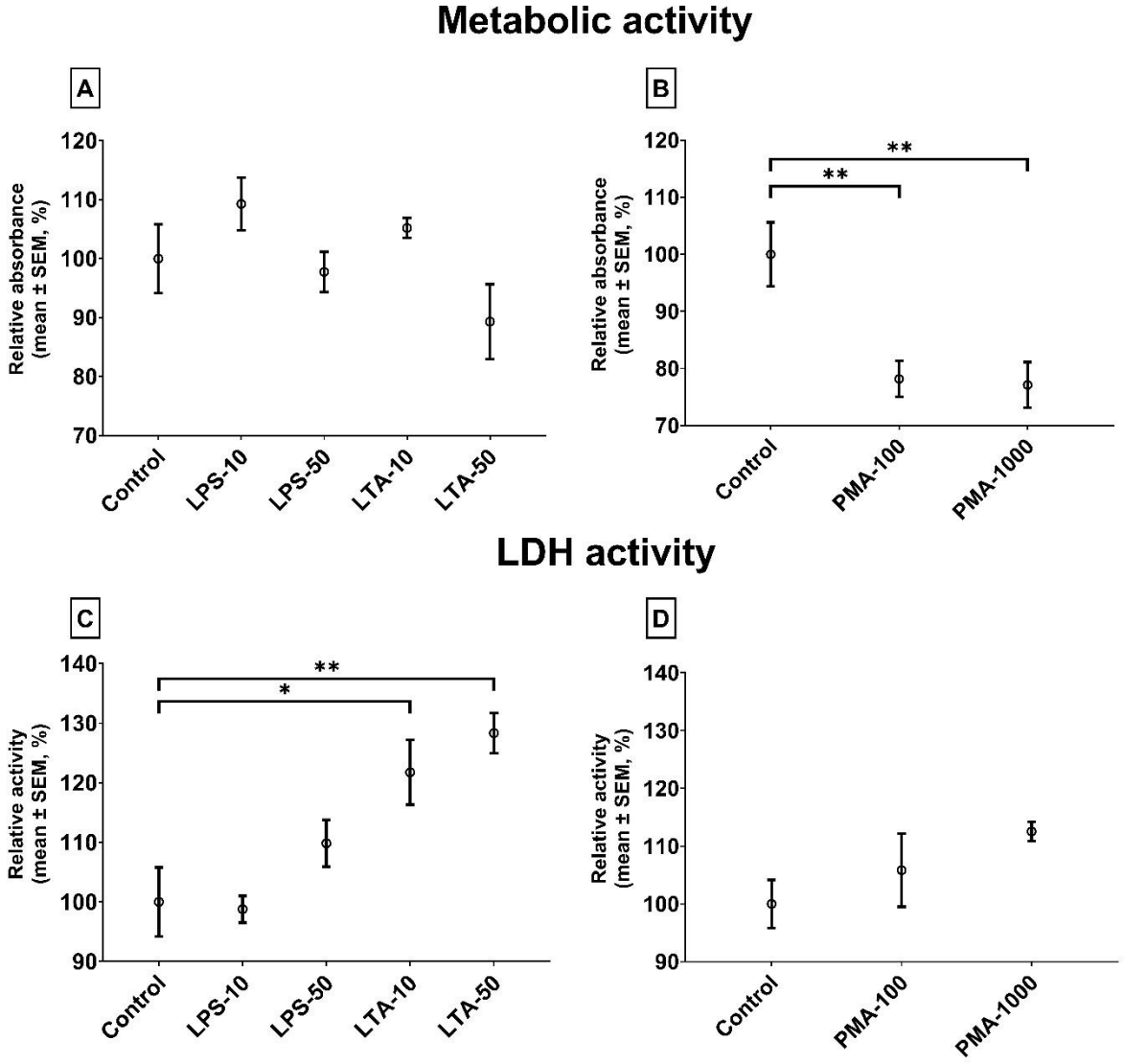

Figure 8. Meanplots showing the changes in metabolic activity (A and B) and extracellular lactate dehydrogenase (LDH) activity (C and D) of the hepatic cell cultures. Results are expressed as mean ± SEM in relative values where 100% is the mean of the corresponding control group. Graphs A and C show the results from the first part of the study (LPS/LTA), B and D show the results from the second part of the study (PMA) with the corresponding control groups. LPS-10/50 = 10 and 50 µg/mL concentrations of lipopolysaccharide from *Escherichia coli* (O55:B5); LTA-10/50 = 10 and 50 µg/mL concentrations of lipoteichoic acid from *Staphylococcus aureus*; PMA-100/1000 = 100 and 1000 ng/mL concentrations of phorbol myristate acetate. Control groups received none of the treatments. $n_{LPS/LTA} = 6$, $n_{PMA} = 10$ for metabolic activity and 5 for LDH activity. * $p<0.05$; ** $p<0.01$

The IL-6 concentration was significantly elevated in the culture media after 1000 ng/mL PMA ($p=0.029$) treatment (Figure 9/B). The concentrations of CXCLi2 were significantly increased by 50 µg/mL LTA ($p=0.013$, Figure 9/C) and 1000 ng/mL PMA ($p=0.029$) treatment ($p=0.036$, Figure 9/D).

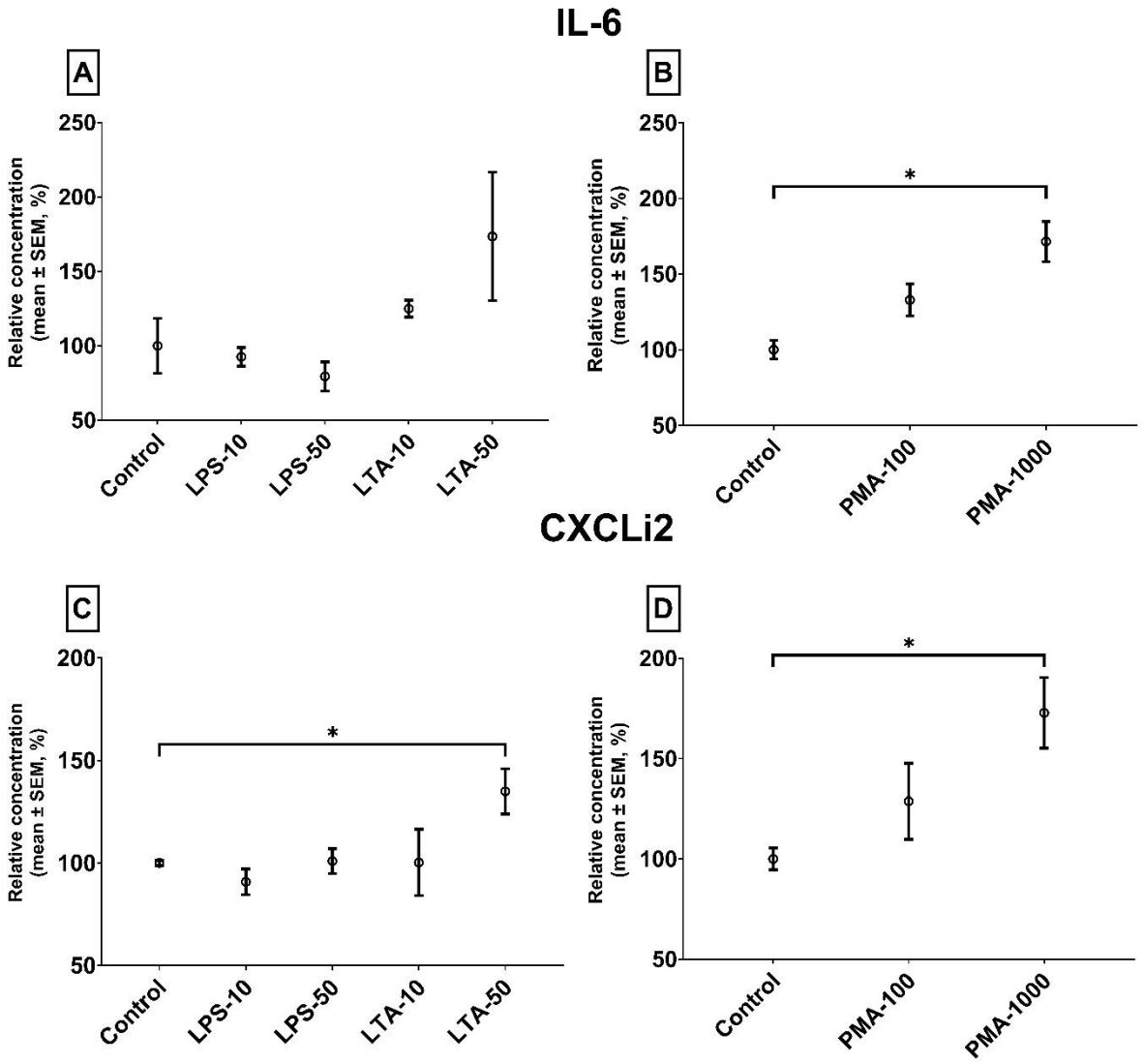

Figure 9. Meanplots showing the changes in interleukin (IL)-6 (A and B) and CXCLi2 (C and D) concentrations of the hepatic cell cultures. Results are expressed as mean ± SEM in relative values where 100% is the mean of the corresponding control group. Graphs A and C show the results from the first part of the study (LPS/LTA), B and D show the results from the second part of the study (PMA) with the corresponding control groups. LPS-10/50 = 10 and 50 µg/mL concentrations of lipopolysaccharide from *Escherichia coli* (O55:B5); LTA-10/50 = 10 and 50 µg/mL concentrations of lipoteichoic acid from *Staphylococcus aureus*; PMA-100/1000 = 100 and 1000 ng/mL concentrations of phorbol myristate acetate. Control groups received none of the treatments. $n_{LPS/LTA} = 6$, $n_{PMA} = 5$. * $p<0.05$

6.2 Study I.: Effects of Cath-2 on a primary hepatic cell culture

Metabolic activity was significantly decreased in the samples treated with both concentrations of Cath-2 (Cath-low and Cath-medium) compared to the Control, by 23,72% and 58,97%, respectively, as seen in Hiba! A hivatkozási forrás nem található.**10/A** (p=0.004, p <0.001, respectively). LTA or PMA exposure did not affect significantly the metabolic activity of the cells when compared to the Control.A

The extracellular LDH activities were increased in the medium of the cells treated with both 5 nmol/mL and 10 nmol/mL Cath-2 (Cath-low and Cath-medium) compared to Control (p<0.001 for both cases, **Figure 10/B**). LDH activity in the cultures that were exposed to LTA showed no significant change compared to the Control, while PMA decreased the LDH activity (p=0.045). With regards to the LTA-exposed cells that were treated with Cath-2, the treatment with the lower dose of the peptide (LTA+Cath-low) showed a significant increase in LDH levels when compared to the LTA-only condition (p=0.045). PMA+Cath-low increased the LDH activity compared to the group that was treated only with PMA (p<0.001; **Figure 10/B**).

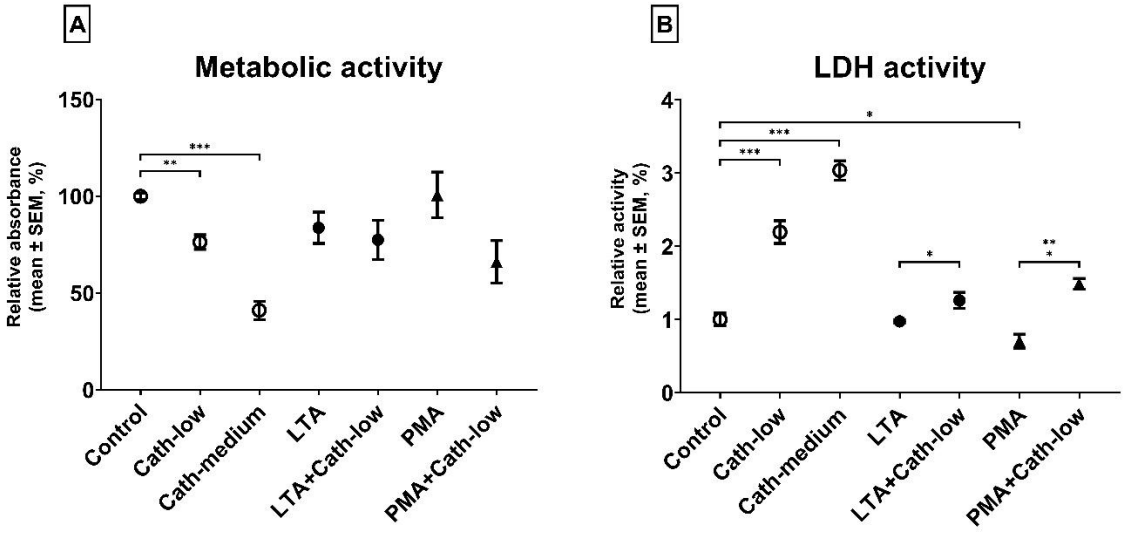

Figure 10. Meanplots showing the changes in metabolic activity (**A**) and extracellular lactate dehydrogenase (LDH) activity (**B**) of the hepatic cell cultures. Results are expressed as mean ± SEM in relative values where 100% is the mean of the control group. Cath-low /10 = 5 and 10 nmol/mL concentrations of chicken cathelicidin-2; LTA = 50 µg/mL concentration of lipoteichoic acid from *Staphylococcus aureus*; PMA = 1000 ng/mL concentration of phorbol myristate acetate. Control groups received none of the treatments. n = 6. * p<0.05; ** p<0.01; *** p<0.001

There was no significant elevation in IFN-γ levels when treated with the lower concentration of Cath-2 (Cath-low), however, as seen in Figure 11/A, cells treated with the

higher dose of the peptide (Cath-medium) showed higher levels of IFN-γ than the Control ($p<0.001$). Compared to the Control, the cultures exposed to LTA showed higher levels of IFN-γ ($p<0.001$). The LTA-exposed cells treated with Cath-2 (LTA+Cath-low) showed a decrease in IFN-γ levels in comparison with the LTA-only condition ($p=0.001$). PMA did not affect the IFN-γ production of the cells (**Figure 11/A**).

As seen in **Figure 11/B**, both Cath-2 concentrations (Cath-low and Cath-medium) significantly decreased the levels of M-CSF ($p=0.031$, $p<0.001$, respectively) compared to the Control. LTA also showed a depressing effect on the concentrations of this molecule ($p=0.004$) compared to the Control, and Cath-2 (LTA+Cath-low) further decreased the levels of M-CSF compared to the group that only received LTA ($p=0.003$). There was a significant increase in the PMA-treated cultures ($p=0.003$) compared to the Control, and in comparison with this group, Cath-2 (PMA+Cath-low) decreased the concentration of M-CSF ($p=0.001$, $p<0.001$, respectively; **Figure 11/B**).

Cultures treated with the lower (Cath-low) and the higher dose (Cath-medium) of Cath-2 had significantly increased levels of CXCLi2 compared to the Control ($p=0.028$, $p<0.001$, respectively, **Figure 11/C**). LTA or PMA did not significantly alter the CXCLi2 levels. In the LTA+Cath-low group, the CXCLi2 concentrations did not differ from the LTA group significantly, however, in the PMA+Cath-low group, the CXCLi2 levels were significantly elevated compared to the group that only received PMA ($p=0.002$, **Figure 11/C**).

IL-10 cytokine levels followed patterns mostly similar to CXCLi2 (Figure 11/D). Compared to the Control, cells treated with only the higher concentration of Cath-2 (Cath-medium) showed an increase in IL-10 levels ($p<0.001$). There were no significant differences in IL-10 levels between the Control and the cell cultures exposed to only LTA or PMA. However, a significant decrease in IL-10 levels was observed in the LTA-exposed cells that were treated with Cath-2 (LTA+Cath-low) compared to the LTA group ($p=0.033$, **Figure 11/D**).

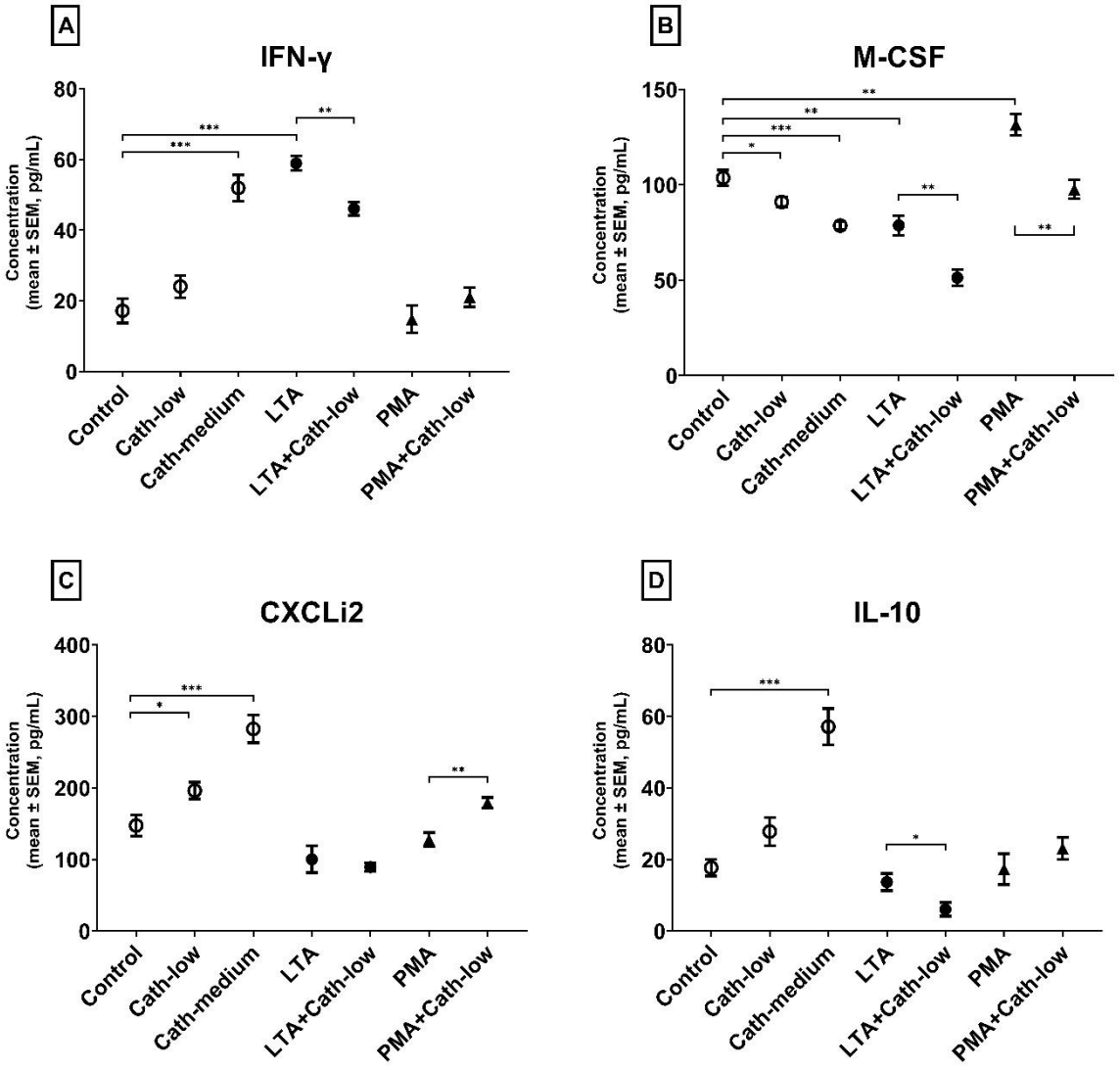

Figure 11. Meanplots showing the changes in concentrations of interferon (IFN)-γ (**A**), macrophage colony-stimulating factor (M-CSF, **B**), CXCLi2 (**C**), and interleukin (IL)-10 (**D**) of the hepatic cell cultures. Results are expressed as mean ± SEM in pg/mL. Cath-low /10 = 5 and 10 nmol/mL concentrations of chicken cathelicidin-2; LTA = 50 μg/mL concentration of lipoteichoic acid from *Staphylococcus aureus*; PMA = 1000 ng/mL concentration of phorbol myristate acetate. Control groups received none of the treatments. n = 6. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

The H_2O_2 concentrations were elevated after both Cath-2 concentrations (Cath-low and Cath-medium) ($p=0.001$, $p<0.001$, respectively). LTA also significantly increased the H_2O_2 concentrations ($p<0.001$), and the lower concentration of Cath-2 (LTA+Cath-low) decreased it compared to the LTA group ($p=0.002$). The H_2O_2 concentration was increased after the PMA treatment ($p=0.009$, **Figure 12/A**).

The higher dose of Cath-2 (Cath-medium) decreased the concentration of MDA ($p=0.006$). Neither LTA nor PMA changed the MDA levels significantly (**Figure 12/B**).

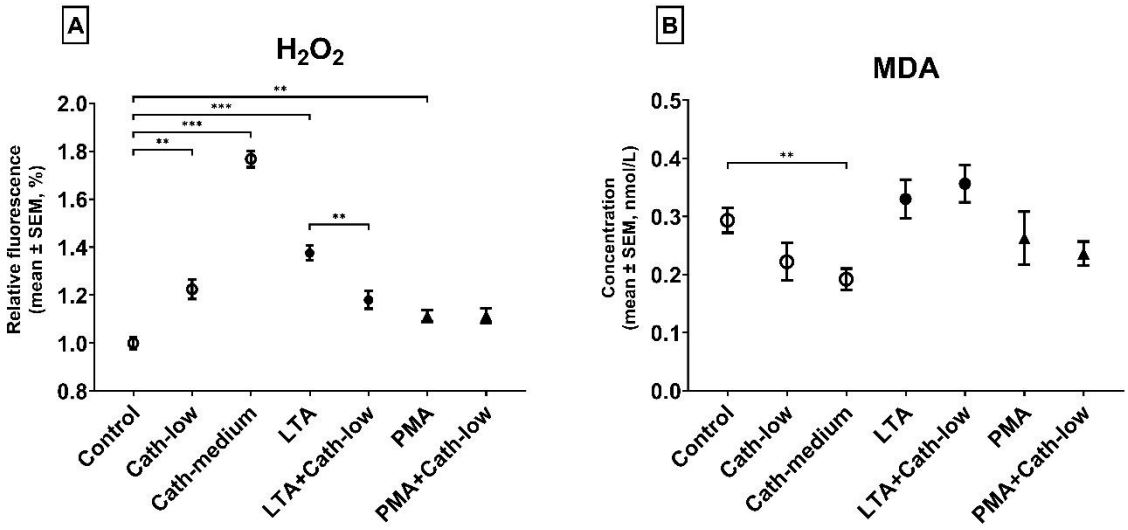

Figure 12. Meanplots showing the changes in extracellular H_2O_2 levels (**A**) and malondialdehyde (MDA) concentrations (**B**) of the hepatic cell cultures. Results are expressed as mean ± SEM, in the case of H_2O_2 levels in relative values where 100% is the mean of the control group and in nmol/mL in the case of MDA. Cath-low /10 = 5 and 10 nmol/mL concentrations of chicken cathelicidin-2; LTA = 50 µg/mL concentration of lipoteichoic acid from Staphylococcus aureus; PMA = 1000 ng/mL concentration of phorbol myristate acetate. Control groups received none of the treatments. n = 6. ** $p<0.01$; *** $p<0.001$

6.3 Study II: Effects of IDR-1002 on a primary hepatic cell culture

No significant changes in metabolic activity were observed either after IDR-1002, LTA, or combination treatments (**Figure 13/A**). IDR-1002 or LTA alone did not alter the extracellular LDH activity, however a significant increase was detected after treatment with LTA and 90 µg/mL concentration of IDR-1002 together compared to the LTA group ($p=0.015$, **Figure 13/B**).

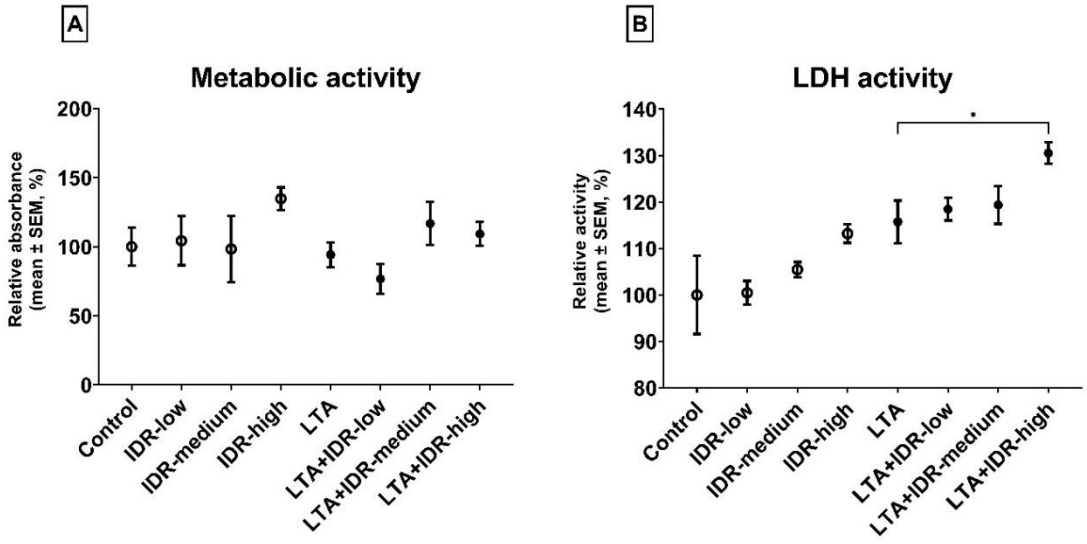

Figure 13. Meanplots showing the changes in metabolic activity (**A**) and extracellular lactate dehydrogenase (LDH) activity (**B**) of the hepatic cell cultures. Results are expressed as mean ± SEM in relative values where 100% is the mean of the control group. IDR-low/medium/high = 10, 30 and 90 µg/mL concentrations of innate defense regulator-1002; LTA = 50 µg/mL concentration of lipoteichoic acid from *Staphylococcus aureus*. Control groups received none of the treatments. n = 6.

* p<.0.05

After IDR-1002 treatment, IL-6 levels were decreased (IDR-low: p=0.030, IDR-medium: p=0.032, IDR-high: p=0.004, **Figure 14/A**), as well as IL-10 concentrations (IDR-low: p=0.009, IDR-high: p=0.002, **Figure 14/B**).

LTA increased the concentrations of CXCLi2 (p=0.027, **Figure 14/C**), IL-6 (p=0.016, Figure 14/A), and IFN-γ (p=0.009, **Figure 14/E**). These elevations were then decreased by IDR-1002, in the case of CXCLi2 by 30 and 90 µg/mL (p=0.019, p = 0.016, respectively, **Figure 14/C**), IL-6 by 10, 30 and 90 µg/mL (p=0.004, p=0.016, p=0.004, respectively, **Figure 14/A**), and IFN-γ by 30 and 90 µg/mL of IDR-1002 (p=0.015, p=0.002, respectively, **Figure 14/E**).

IL-10 and IL-16 concentrations were decreased compared to LTA after treatment with 30 and 90 µg/mL IDR-1002 (IL-10: p=0.002 in both cases, Figure 14/B, IL-16: p=0.002, p=0.005, respectively, **Figure 14/D**).

M-CSF levels were elevated after treatment with 10, 30, 90 µg/mL of the peptide (p=0.015, p=0.004, p=0.009, respectively, **Figure 14/G**), and RANTES levels after treatment with 90 µg/mL (p=0.002, **Figure 14/F**). LTA also increased RANTES concentrations (p=0.002), and IDR-1002 further elevated it compared to LTA (30 and 90 µg/mL: p=0.002 in both cases, **Figure 14/F and G**). M-CSF levels were also increased in the combination groups compared to LTA (LTA+IDR-medium: p=0.009, LTA+IDR-high: p=0.004, **Figure 14/G**).

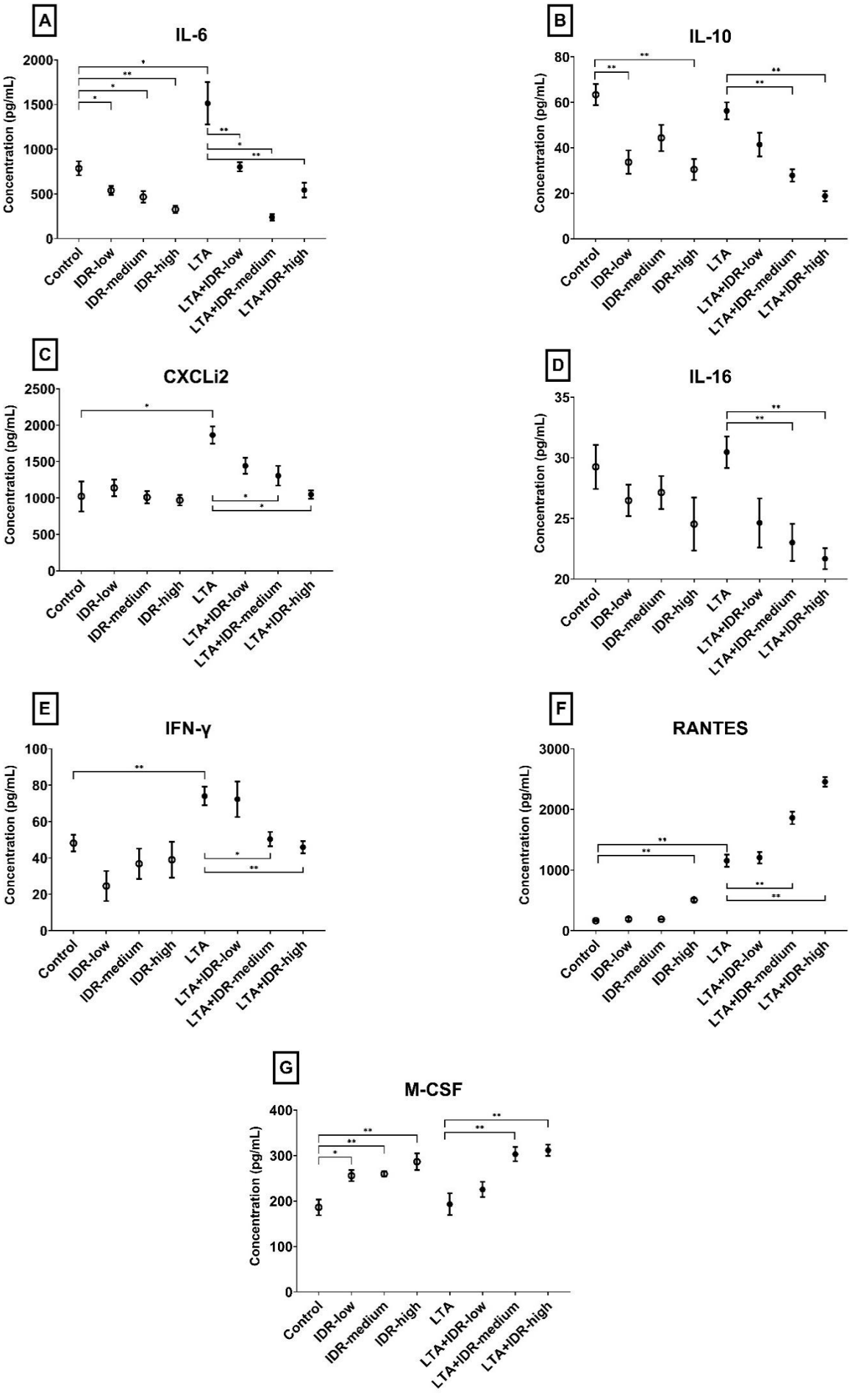
A
IL-6
Concentration (pg/mL)
2000
1500
1000
500
0
Control
IDR-low
IDR-medium
IDR-high
LTA
LTA+IDR-low
LTA+IDR-medium
LTA+IDR-high
B
IL-10
Concentration (pg/mL)
80
60
40
20
0
C
CXCLi2
Concentration (pg/mL)
2500
2000
1500
1000
500
0
D
IL-16
Concentration (pg/mL)
35
30
25
20
E
IFN-γ
Concentration (pg/mL)
100
80
60
40
20
0
F
RANTES
Concentration (pg/mL)
3000
2000
1000
0
G
M-CSF
Concentration (pg/mL)
400
300
200
100
0

Figure 14. Meanplots showing the changes in concentrations of interleukin (IL)-6 (**A**), IL-10 (**B**), CXCLi2 (**C**), IL-16 (**D**), interferon (IFN)-γ (**E**), RANTES (**F**) and macrophage colony stimulating factor (M-CSF (**G**), of the hepatic cell cultures. Results are expressed as mean ± SEM in pg/mL. IDR-low/medium/high = 10, 30 and 90 μg/mL concentrations of innate defense regulator-1002; LTA = 50 μg/mL concentration of lipoteichoic acid from *Staphylococcus aureus*. Control groups received none of the treatments. n = 6. * p<.0.05, **p<0.01

In addition, a strong positive correlation was found between RANTES and IFN-γ in the groups LTA+IDR-medium and -high, between RANTES and IL-16 in groups IDR-high and LTA+IDR-high, and interestingly, between RANTES and IL-10 in groups LTA+IDR-medium and -high. There was a negative correlation between M-CSF and CXCLi2 in the LTA+IDR-medium group, and a mentionable, but not significant positive correlation between M-CSF and IL-10 in the group LTA+IDR-medium. Details and plots are included as **Supplementary Figures 1-10 in chapter 12 ("Supplementary material")**.

Regarding the redox homeostasis, 30 μg/mL IDR-1002 decreased the H_2O_2 concentration (p=0.03, **Figure 15/B**), and all three concentrations of the peptide elevated the Nrf2 concentrations (p=0.014, p=0.032, p=0.016, respectively, **Figure 15/A**). Protein carbonyl levels were also increased after treatment with all concentrations (p=0.004 in all cases, **Figure 15/C**). LTA similarly elevated Nrf2 (p=0.016), and in combination, 10 μg/mL IDR-1002 decreased it (p=0.032). H_2O_2 levels were also decreased by the LTA+IDR-high treatment compared to LTA (p=0.026, **Figure 15/A**). Positive correlation was found between Nrf2 and PC in the IDR-low group as well, and a positive, but not significant one between Nrf2 and H_2O_2 in LTA+IDR-high (**Supplementary Figures 11-12 in chapter 12 ["Supplementary material"]**).

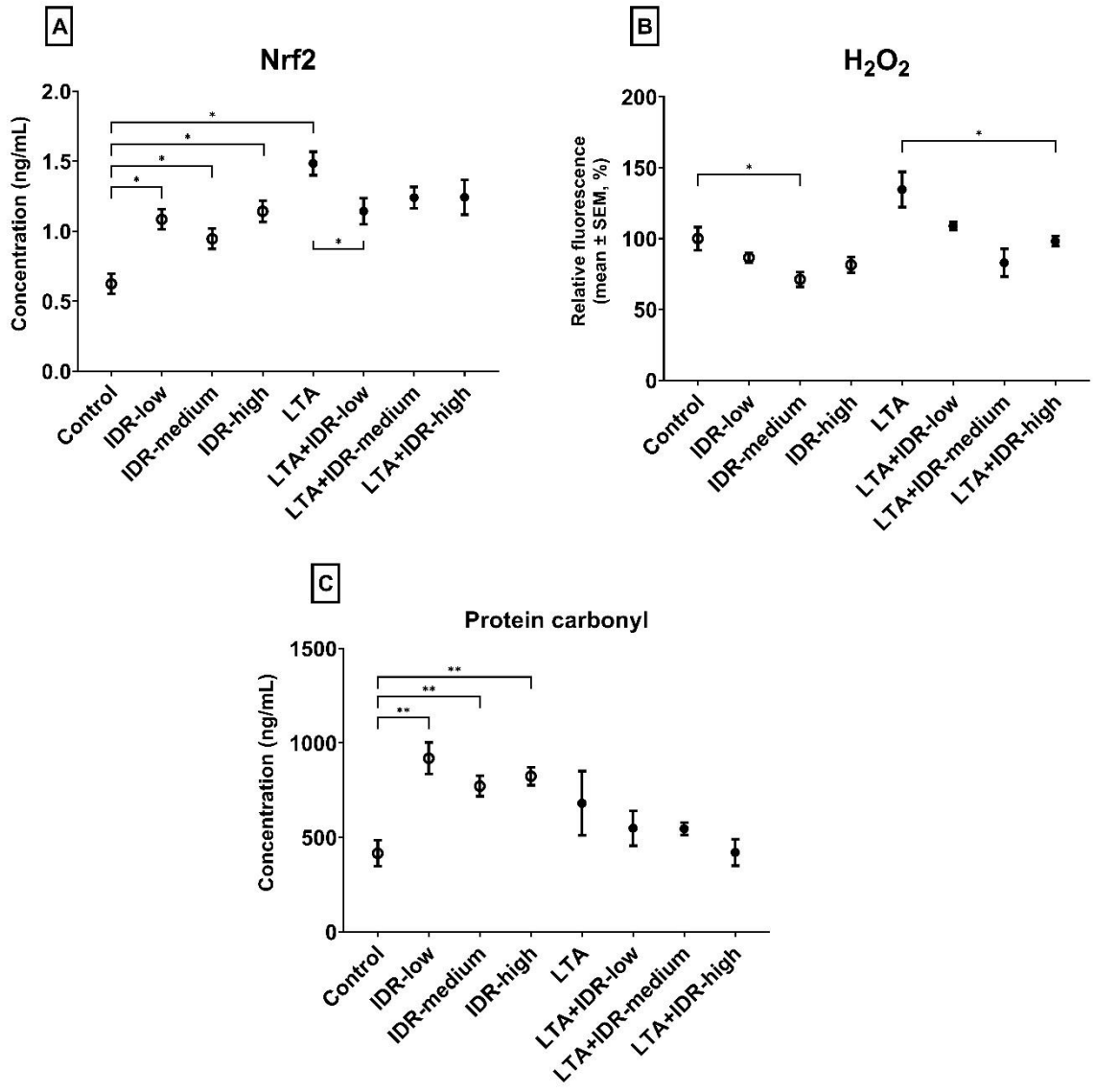

Figure 15. Meanplots showing the changes in nuclear factor erythroid 2-related factor (Nrf2, **A**) concentrations, H_2O_2 levels (**B**), and protein carbonyl concentrations (**C**) of the hepatic cell cultures. Results are expressed as mean ± SEM as relative values where 100% is the mean of the control group in the case of H_2O_2 levels, and in pg/mL in the case of Nrf2 and protein carbonyl. IDR-low/medium/high = 10, 30 and 90 µg/mL concentrations of innate defense regulator-1002; LTA = 50 µg/mL concentration of lipoteichoic acid from *Staphylococcus aureus*. Control groups received none of the treatments. n = 6. * p<.0.05, **p<0.01

6.4 Study III: Effects of Cath-2 on an intestinal explant culture

The metabolic activity of the explants was elevated after treatment with 25 nmol/mL Cath-2 (Cath-high; p=0.010, **Figure 16/A**). No other treatment influenced the metabolic activity or the LDH activity of the explants in a significant manner (**Figure 16/A and B**).

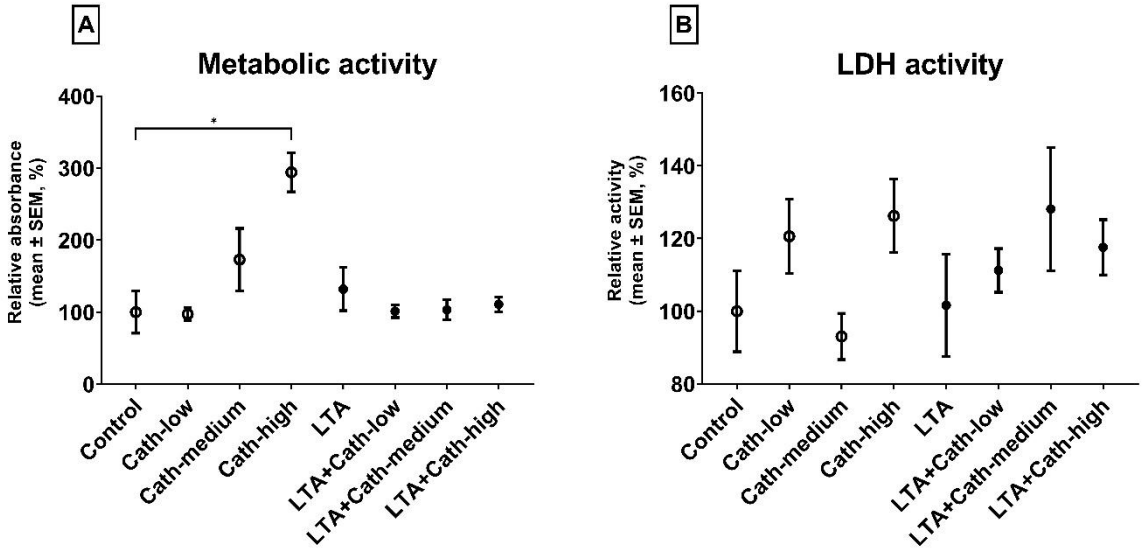

Figure 16. Meanplots showing the changes in metabolic activity (**A**) and extracellular lactate dehydrogenase (LDH) activity (**B**) of the intestinal explants. Results are expressed as mean ± SEM in relative values where 100% is the mean of the control group. Cath-low/medium/high = 5, 10 and 25 nmol/mL concentrations of chicken cathelicidin-2; LTA = 10 µg/mL concentration of lipoteichoic acid from *Staphylococcus aureus*. Control groups received none of the treatments. n = 6. * p<.0.05

IL-6 concentration increased following 10 nmol/mL concentration Cath-2 (Cath-medium), and LTA treatment (p=0.016 in both cases, **Figure 17/A**) compared to Control. The concomitant addition of 25 nmol/mL concentration of Cath-2 to LTA-exposed cells (LTA+Cath-high) decreased cellular IL-6 production compared to the LTA group (p=0.029, **Figure 17/A**).

CXCLi2 levels were increased after treatment with 5, 10, 25 nmol/mL of Cath-2 (Cath-low, Cath-medium, and Cath-high), and LTA (p=0.019, p=0.027, p=0.014, p=0.016, respectively, **Figure 17/B**) compared to the non-treated controls. Furthermore, the combinatory administration of LTA and 25 nmol/mL Cath-2 (LTA+Cath-high) further increased the concentration of CXCLi2 compared to the already elevated production in the LTA group (p=0.016, **Figure 17/B**).

25 nmol/mL Cath-2 (Cath-high), and LTA treatment increased the production of IL-2 in with comparison with the Control group (p=0.022, p=0.009, respectively, **Figure 17/C**). Combining LTA with 10 and 25 nmol/mL concentrations of Cath-2 (LTA+Cath-medium and

LTA+Cath-high) decreased IL-2 levels compared to the LTA group (p=0.013 in both cases, **Figure 17/C**).

The concentration of IFN-γ was increased and IL-10 was decreased after LTA treatment (p=0.005, p=0.031, respectively, **Figure 17/D and E**), which then lead to an increase in the ratio of IFN-γ/IL-10 (p=0.036, **Figure 17/F**). The combinatory exposure to LTA and 25 nmol/mL Cath-2 (LTA+Cath-high) elevated IL-10 levels compared to LTA (p=0.024, **Figure 17/E**), but had no influence on IFN-γ concentrations. However, the ratio of IFN-γ/IL-10 was decreased by the concomitant application of 5, 10 and 25 nmol/mL of Cath-2 (LTA+Cath-low, LTA+Cath-medium, and LTA+Cath-high) compared to the group receiving only LTA (p=0.048, p=0.024, p=0.028, respectively, **Figure 17/F**).

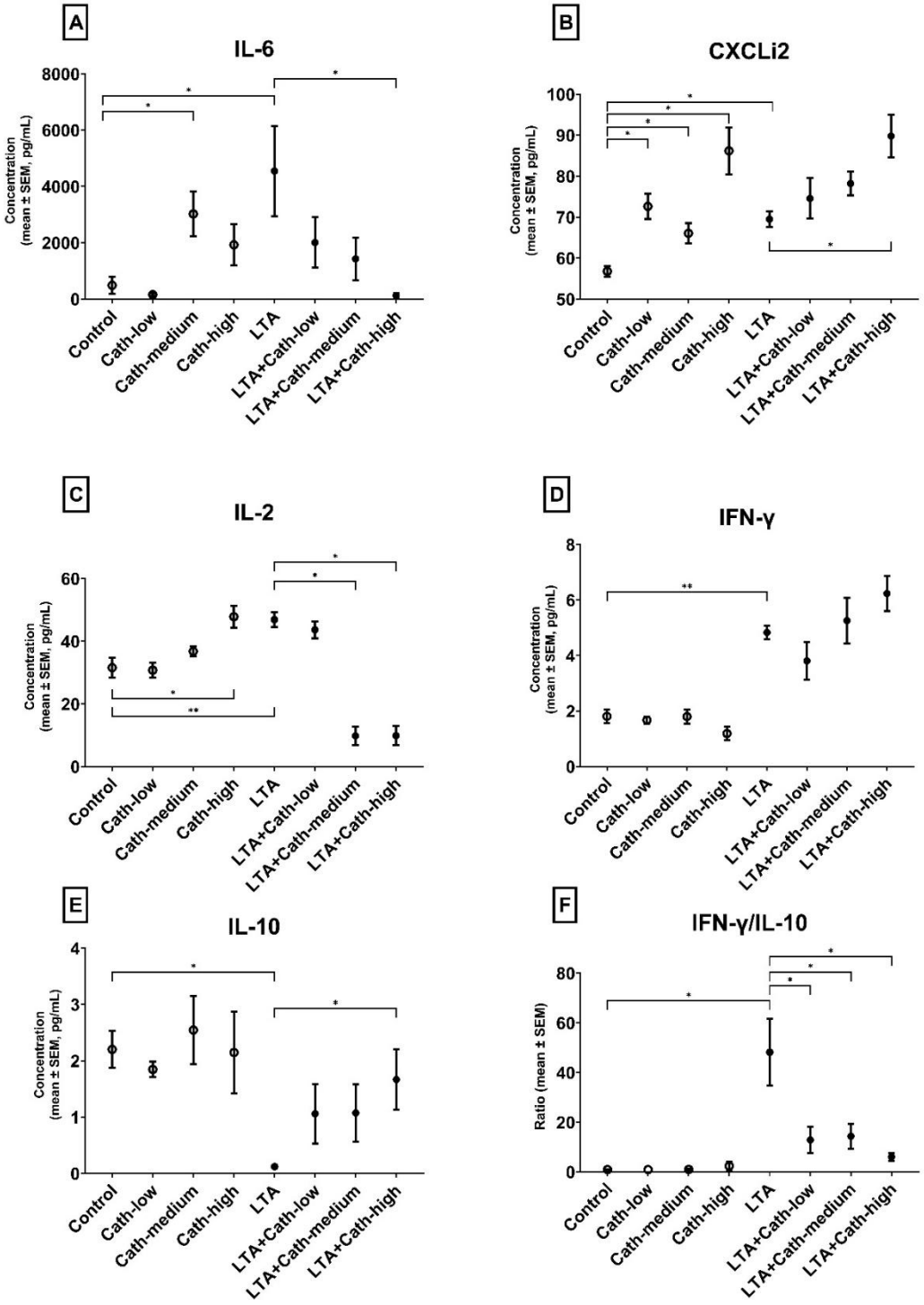

Figure 17. Meanplots showing the changes in concentrations of interleukin (IL)-6 (**A**), CXCLi2 (**B**), IL-2 (**C**), interferon (IFN)-γ (**D**), IL-10 (**E**) and the IFN-γ/IL-10 ratio (**F**) of the intestinal explants. Results are expressed as mean ± SEM in pg/mL, and in the case of IFN-γ/IL-10 ratio, as the ratio of the two concentrations. Cath-low/medium/high = 10, 30 and 90 µg/mL concentrations of chicken cathelicidin-2; LTA = 10 µg/mL concentration of lipoteichoic acid from *Staphylococcus aureus*. Control groups received none of the treatments. n = 6. * p<.0.05, **p<0.01

6.5 Study IV: Effects of IDR-1002 on an intestinal explant culture

No significant changes were observed regarding the metabolic activity of the explants (**Figure 18/A**). LDH activity was decreased in the group treated with 30 µg/mL IDR-1002 compared to the control (IDR-medium; p=0.029; **Figure 18/B**).

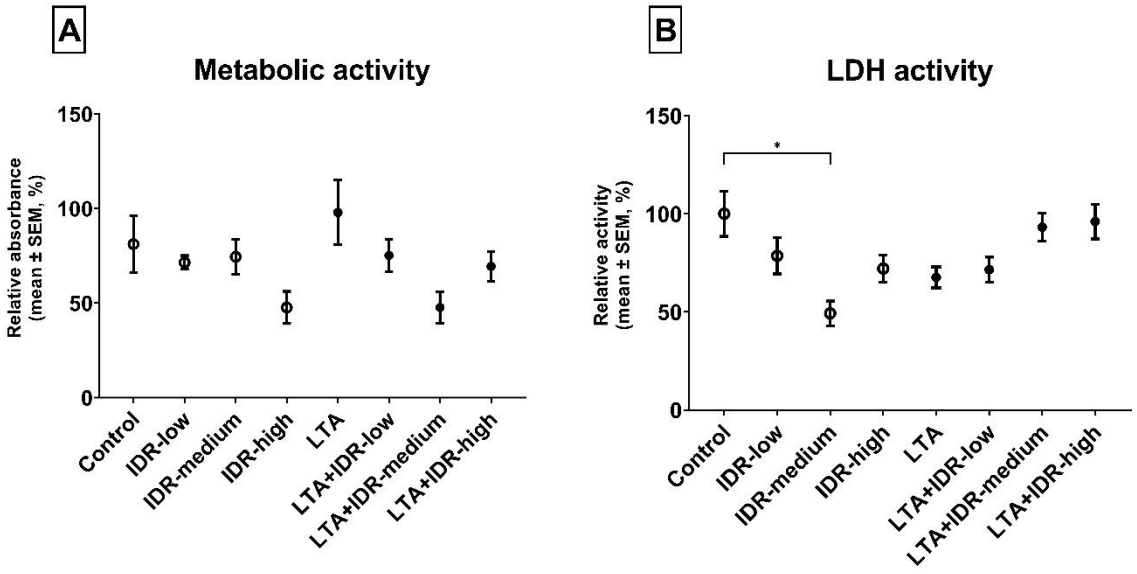

Figure 18. Meanplots showing the changes in metabolic activity (**A**) and extracellular lactate dehydrogenase (LDH) activity (**B**) of the intestinal explants. Results are expressed as mean ± SEM in relative values where 100% is the mean of the control group. IDR-low/medium/high = 10, 30 and 90 µg/mL concentrations of innate defense regulator-1002; LTA = 10 µg/mL concentration of lipoteichoic acid from *Staphylococcus aureus*. Control groups received none of the treatments. n = 6. * p<.0.05

It was found that treatment with 10, 30, and 90 µg/mL of IDR-1002 (IDR-low, IDR-medium, IDR-high; p=0.035; 0.023; 0.036, respectively) and LTA (p=0.019) resulted in significantly elevated IL-2 levels compared to the control group. IDR-1002 applied with LTA at a concentration of 10 µg/mL (LTA+IDR-low) significantly decreased (p=0.028) the IL-2 concentration of culture media compared to the group exposed to LTA alone (**Figure 19/A**).

The obtained results show that the sole treatment with 30 µg/mL IDR-1002 (IDR-medium) and with LTA resulted in elevated (p=0.017; 0.006, respectively) CXCLi2 levels compared to the control group. Further, all concentrations of IDR-1002 (10, 30, 90 µg/mL IDR-1002) in combination with LTA (LTA+IDR-low, LTA+IDR-medium, LTA+IDR-high) caused significantly lower (p=0.004; 0.002; 0.005, respectively) CXCLi2 concentrations compared to the group with LTA alone (**Figure 19/B**).

Based on the obtained results LTA alone, and LTA supplemented with 90 μg/mL IDR-1002 (LTA+IDR-high) significantly increased (p=0.01; 0.041, respectively) the measured RANTES concentrations compared to the control group. In addition, 10 and 30 μg/mL concentrations of IDR-1002 when administered together with LTA (LTA+IDR-low, LTA+IDR-medium) significantly decreased (p=0.016; 0.029, respectively) the levels of RANTES compared to explants treated with LTA (**Figure 19/C**).

The treatment with LTA significantly increased (p=0.017) the concentration of IFN-γ produced by the explants compared to the control group, as well as all combination groups showed elevated IFN-γ concentrations (LTA+IDR-low, LTA+IDR-medium, LTA+IDR-high; p=0.004; 0.005; 0.005, respectively). Furthermore, a significant elevation (p=0.009) was observed when the LTA-exposed group was supplemented with IDR-1002 treatment at a concentration of 90 μg/mL (LTA+IDR-high) compared to the solely LTA-treated group (p=0.019; **Figure 19/D**).

IL-10 concentrations were decreased after treatment with LTA and 10 μg/mL IDR-1002 (LTA+IDR-low; p=0.011) and increased after treatment with LTA and 90 μg/mL of IDR-1002 (LTA+IDR-high; p=0.005) compared to control. Furthermore, supplementation of LTA with 90 μg/mL of IDR-1002 (LTA+IDR-high) elevated IL-10 concentrations compared to the LTA group (p=0.007; **Figure 19/E**).

The ratio of IFN-γ/IL-10 was elevated in the LTA-treated group (p=0.005) and also in the LTA group supplemented with 10 μg/mL IDR-1002 (LTA+IDR-low; p=0.011, p=0.007, respectively). The group with LTA and 90 μg/mL IDR-1002 (LTA+IDR-high) decreased the ratio compared to the LTA group (p=0.008; **Figure 19/F**).

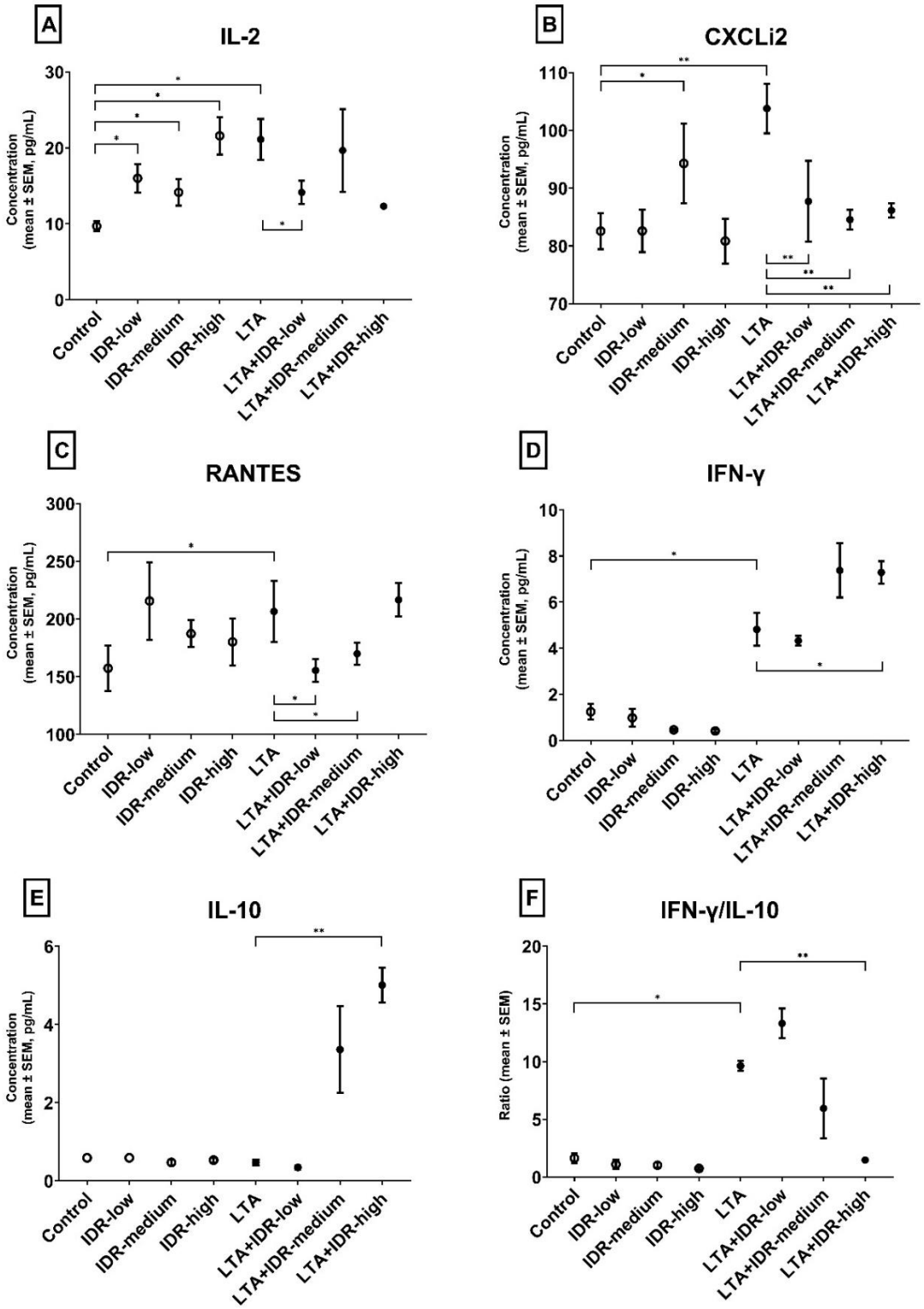

Figure 19. Meanplots showing the changes in concentrations of interleukin (IL)-2 (A), CXCLi2 (B), RANTES (C), interferon (IFN)-γ (D), IL-10 (E) and the IFN-γ/IL-10 ratio (F) of the intestinal explants. Results are expressed as mean ± SEM in pg/mL, and in the case of IFN-γ/IL-10 ratio, as the ratio of the two concentrations. IDR-low/medium/high = 10, 30 and 90 µg/mL concentrations of innate defense regulator-1002; LTA = 10 µg/mL concentration of lipoteichoic acid from *Staphylococcus aureus*. Control groups received none of the treatments. n = 6. * p<.0.05, **p<0.01

7 Discussion

The importance of antimicrobial resistance and the effect it has on the livestock industry has become unequivocal in recent years. The treatment of intestinal bacterial infections and the consequent inflammatory processes in chickens is facing more and more difficulties, leading to detrimental economic outcomes such as decreased growth rates, increased mortality, or reduced egg production [163]. However, AMPs may provide a solution to this problem e.g. by interacting with several cell types either in the intestinal wall, or in the liver, and therefore modulating the inflammation and eliminating pathogenic bacteria of GI origin. For testing the immunomodulatory effects of these molecules, efficient and reliable models are needed to be developed. The chicken hepatic cell cultures and the intestinal explant technique used in this study have proven to be effective in tracking the immune response after treatment with the Cath-2 and IDR-1002 peptides, the pro-inflammatory LTA and PMA, or those combined. Summary of these results can be viewed in **Table 6**. We believe that our results provide novel and suitable information concerning the immunomodulatory role of AMPs in the field of enteric and hepatic inflammation.

Table 6.: Summary of the results

AMP	Model	Cell viability	Pro-inflammatoric effects	Anti-inflammatoric effects	Redox parameters
Cath-2	Chicken hepatocyte – non-parenchymal cell co-culture	↓ (concentration dependence)	IFN-γ, CXCLI2 ↑ (M-CSF ↓)	*IL-10* ↑ IFN-γ ↓ M-CSF ↓	H_2O_2 ↑ H_2O_2 ↓ MDA ↓
IDR-1002	Chicken hepatocyte – non-parenchymal cell co-culture	no change (excluding: together with LTA)	IL-10 ↓ RANTES ↑	*IL-6* ↓ CXCLi2, IFN-γ ↓ M-CSF ↑	Nrf2 ↑, with LTA ↓ H_2O_2 ↓ PC ↑
Cath-2	Chicken hepatocyte – non-parenchymal cell co-culture	no change	IL-6, CXCLi2, IL-2 ↑	IL-6, IL-2 ↓ IL-10 ↑ IFN-γ/IL-10 ↓	-
IDR-1002	Chicken intestinal explant	no change	IL-2, CXCLi2 ↑	IL-2, CXCLi2, RANTES ↓ IFN-γ/IL-10 ↓	-

7.1 Preliminary study: induction of inflammation

To investigate the effect certain compounds have on the immune response, it is inevitable to have an efficient inflammatory model suitable for the investigated cell cultures. The goal of this Preliminary study was to find molecules that would be fit to induce inflammatory response in our chicken hepatic cell cultures.

There is only limited data available related to this issue in cell cultures of chicken origin; however, it would be an essential basis for future studies concerning the *in vitro* testing of immunomodulatory agents. The applied primary hepatocyte – non-parenchymal cell co-cultures of chicken origin have already been used in previous studies to investigate the cellular effects of acute heat stress and T-2 toxin [161, 164]. Furthermore, even inflammatory models were designed using similar cultures of porcine origin [165]. The inclusion of the non-parenchymal cell fraction in co-cultures at cell ratio 6:1 (hepatocytes to non-parenchymal cells) refers to a mild hepatic inflammatory state with moderate intrahepatic macrophage migration [161], enabling the investigation of the link between the inflammatory and stress response.

In the first part of the present study, we intended to examine the effects of bacterial endotoxins (LPS of Gram-negative and LTA of Gram-positive origin) as traditional proinflammatory agents. As the applied endotoxin treatments except LTA applied in 50 µg/mL could not induce proinflammatory cytokine production in the cell cultures used, further potential PAMPs (ETxB from *Escherichia coli*, flagellin from *Salmonella* Typhimurium, PMA and poly I:C) were screened next. From these compounds, as mentioned before, in this work only the results of PMA treatment are discussed, as only LTA and PMA were used in the latter studies.

It was aimed to monitor how the applied proinflammatory agents affected the metabolic activity and membrane damage of the cultured cells. It was found that LTA elevated the extracellular LDH levels, indicating the occurrence of membrane damage. Furthermore, PMA treatment decreased the metabolic activity of the cell cultures, demonstrating a metabolically depressed state because of the harmful effect of this compound.

The pro-inflammatory effects of our candidate molecules were screened by measuring the concentrations of IL-6 and CXCLi2 in culture media. Notwithstanding that some other pro- and anti-inflammatory mediators should be investigated in further studies, monitoring the hepatocellular IL-6 and CXCLi2 response provided sufficient initial data concerning the pro-inflammatory action of the tested candidates. According to our results, while PMA elevated both IL-6 and CXCLi2 levels, the pro-inflammatory effect of LTA was only proved by the increase in CXCLi2 concentrations, and LPS did not change the production of either cytokine.

Kim et. al described a TLR signal-independent route in which PKC signal transduction can enhance IL-6 production. This mechanism is related to a cytoskeletal regulatory protein

and actin bundling which is essential for the translation of IL-6 mRNA. In accordance with our results, PMA as a direct activator of PKC would serve as a potent agent to activate independent and separate steps of TLR-mediated cytokine production in the absence of a PAMP [158, 159, 166].

It can be stated that LPS did not have a proinflammatory effect in the hepatic cell cultures. The inadequate IL response caused by *Escherichia coli* LPS is a possible result of the deficient signal transduction of chicken TLR4. TLRs specified for extracellular pathogen recognition exclusively activate myeloid differentiation primary response protein 88 (MyD88) dependent pathway which is needed for the prompt activation of Nf-κB. In human TLR-3 and -4 adaptors, double-stranded RNA and LPS can set off interferon production and a so-called delayed or late-phase activation of NF-kappa B via a sovereign MyD88 independent route. The inadequate IL response caused by *Escherichia coli* LPS is possibly related to the deficient signal transduction of chicken TLR4 and the lack of this additional route. This hypothesis was confirmed by Keestra and van Putten by the cited lack of interferon production in response to different *Salmonella* Enteritidis and Gallinarum as well as *Pasteurella multocida* and secondly by the absence of certain TLR4 signal mammalian gene orthologs in chicken [78, 154, 167–169]. This concept is still controversial as two different research groups managed to trigger IFN response with *Escherichia coli* and *Salmonella* Typhimurium in cell cultures of chicken origin [170, 171]; however, only on the level of gene expression. It can be hereby stated that IL-6 and IL-8 protein level elevation could not be induced in cell culture media with LPS from the chicken pathogenic O55:B5 serotype of *Escherichia coli* in accordance with the relatively high tolerance to LPS in avian species [168].

Nonetheless, there is just a handful of published data on the effect of LPS on the secreted IL-6 and CXCLi2/IL-8 concentrations in chicken. These interleukin levels showed only a slight or no increase as opposed to the remarkable elevation of the respective gene expressions detected by the RT-PCR method [172–175]. As both interleukins were measured directly from cell culture media, the differences between the present results and those of studies assessing mRNA levels might arise from post-transcriptional, translational and post-translational regulatory mechanisms. These processes, for example, interleukin mRNA accumulation or degradation by ribonuclease enzymes and PKC-dependent translational regulation of IL-6 level are included in the determination of the final inflammatory cytokine response of the cell [166, 176, 177].

Based on the results of this study, it can be concluded that LTA and PMA are suitable candidates to induce an inflammatory response in the chicken liver cell cultures developed by our research group, and they can be used in future studies involving immunomodulatory agents.

7.2 Study I: Effects of Cath-2 on a primary hepatic cell culture

In Study I., the effects of the AMP Cath-2 were investigated on the hepatocyte – non-parenchymal cell co-culture of chicken origin, for which the inflammatory model was established in the Preliminary study. The first question was whether Cath-2 affects the viability and membrane integrity of chicken hepatic cells, and the second was how their inflammatory state is getting altered.

The main obstacle to the therapeutic use of natural AMPs is their adverse effect on the target organism, such as cytotoxicity, the stimulation of mast cell degranulation, and apoptosis [84, 178–180]. The antimicrobial activity of these peptides can be explained by their interaction with the membranes. It is generally accepted that naturally occurring, positively charged AMPs interact with negatively charged components of the cell membrane and then get inserted into the membrane in a parallel direction, thereby disrupting the continuity of the phospholipid bilayer. This allows the peptide to internalize and exert its intracellular effects [181]. However, high reactivity with the membrane, and thus potent antimicrobial activity may also be associated with toxic effects on the cells of the target organism. For example, LL-37 shows relatively high cytotoxicity not just towards bacterial, but eukaryotic cells as well [182–184].

In our study, the metabolic activity and the membrane integrity of the cells were assessed to determine whether Cath-2 has any unfavorable effects on the host cells. It was found that the AMP decreased the metabolic activity, and at the same time, increased the LDH activity in cell culture media. These changes were seemingly more considerable with the higher dose of the peptide, suggesting a dose-dependent effect of the AMP. According to our results, the metabolic activity was decreased by approximately 60% with the higher dose of cathelicidin-2, which could indicate remarkable cell damage, but with the lower dose, the decrease was only about 20%, which is considered as just a mild metabolic depression [185, 186].

Neither LTA nor PMA affected the metabolic activity or the LDH levels significantly. Exposure to LTA concomitantly with the higher dose of Cath-2 treatment resulted in a significantly decreased metabolic activity. Interestingly, in case of LTA-induced inflammation, the lower dose of Cath-2 did not influence the metabolic activity when compared to the LTA-only condition. This indicates that in the presence of LTA, Cath-2 only has a decreasing effect on the metabolic activity of hepatocyte - non-parenchymal cell co-cultures when applied at higher doses. In line with these results, in the absence of LTA exposure, both Cath-2 treatment conditions showed a dose-dependent increase in LDH levels compared to the control, but with LTA supplementation, this elevation was not considerable. It has been found that Cath-2 binds

and neutralizes negatively-charged endotoxins like LPS and LTA, which plays a major role in its mechanism of action [187]. A model that might explain the data from the metabolic and LDH assays is that the peptide preferentially binds LTA; hence, no off-target cytotoxic effects could be observed in liver cell co-cultures when applying appropriate concentrations. Excessive concentrations of the AMP, however, may result in unbound Cath-2 and the development of off-target effects, as seen in the treatment conditions without LTA-induced inflammation and the LTA-exposed cells treated with a higher dose of the peptide.

In the present study, the concentration of four cytokines and chemokines was aimed to be assessed: CXCLi2 (the most similar chemokine in chickens to the human IL-8 [188]), IL-10, IFN-γ and M-CSF. These mediators are produced by a variety of cell types and have different and diversified mechanisms in influencing the inflammatory response. While CXCLi2 and IFN-γ can be considered as pro-inflammatory cytokines, the effect of M-CSF on the macrophages is much more complex. These molecules together with the anti-inflammatory IL-10 can provide us with an overview of the effects that Cath-2 may have on the immune system [189–192].

The main role of IFN-γ is to activate immune cells, including macrophages, in order to stimulate the immune system to fight infections [193]. Upon activation by this cytokine, macrophages have been found to increase their phagocytic activity and their ROS and RNS production. It was found in our study that co-cultures treated with the higher dose of Cath-2 produced increased levels of IFN-γ compared to the control. Similar results were found for a *Salmo salar* cathelicidin-derived peptide, applied to head kidney leukocytes in the absence of endotoxin-stimulated inflammation, resulting in increased expression of IFN-γ [194]. With regards to the cells exposed to only LTA, IFN-γ was produced at higher levels than the control, indicating a pro-inflammatory effect of LTA. Cells treated with both LTA and 5 nmol/mL of cathelicidin-2 showed a decrease in IFN-γ levels when compared to those treated only with LTA. Based on these data, it could be stated that the lower dose of Cath-2 could contribute to the restoration of the inflammatory homeostasis by successfully alleviating the LTA-triggered IFN-γ release, while the higher peptide concentration elicited pro-inflammatory activity.

CXCLi2 is a pro-inflammatory chemokine implicated with neutrophil (or in case of chickens, heterophil) infiltration into inflamed tissues [188, 195]. Neutrophil/heterophil infiltration is important for clearance of infection, but it should also be noted that these cells mount an aggressive response during inflammation that leads to tissue damage [196]. For this reason, IL-8 release is tightly controlled by anti-inflammatory molecules such as IL-10 [197].

According to our present results, a dose-dependent increase in CXCLi2 levels was found in the cell cultures treated with Cath-2, providing more support for the theory that the role of this peptide in the liver is immunomodulatory rather than solely anti-inflammatory. This finding is in line with other studies that found that human cathelicidins increased the expression and production of IL-8 [198, 199], and chicken cathelicidin-2 induced the transcription of

CXCLi2 [186]. However, in this study, LTA or PMA did not cause significant changes in CXCLi2 levels compared to the control. The extracellular CXCLi2 concentration of cells exposed to LTA together with Cath-2 was not different compared to those only treated with LTA, so the pro-inflammatory effect of the AMP was not observed when it was applied together with LTA. In contrast, combined Cath-2 and PMA exposure caused similar elevation in CXCLi2 levels as the sole application of the AMP.

IL-10 acts indirectly to suppress the expression of pro-inflammatory genes and thereby keeps inflammatory responses regulated [200]. Cells treated with the higher dose of Cath-2 showed a significant increase in IL-10 levels compared to the control, providing further evidence for the immunomodulatory function of Cath-2 in the liver. Previous research has found that IL-10 initiates an important defense mechanism against inflammatory overshoot caused by IL-8, which explains the similarities between CXCLi2 and IL-10 levels [197]. Increases in CXCLi2 levels need to be offset with elevated IL-10 release to maintain inflammatory homeostasis in the liver cells. It should be noted that there were no significant differences between the cells exposed to LTA and the control. There was, however, a slight but significant decrease in IL-10 levels in the cells concomitantly exposed to LTA and the lower dose of Cath-2. The data from the CXCLi2 and IL-10 assays coincide with the results from the cellular metabolism and LDH assays, altogether providing support for the theory that the binding of LTA to Cath-2 results in the loss of its ability to induce cytokine release in chicken hepatocyte - non-parenchymal cell co-cultures.

The role of M-CSF (also known as CSF-1) in inflammation is especially complex. It stimulates the proliferation, differentiation, and activity of the mononuclear phagocyte cell lineage [201]. The biological functions of M-CSF are mediated by a tyrosine kinase receptor called CSF-1R that is expressed on all myeloid cells belonging to the mononuclear phagocytic lineage. Binding of M-CSF to its receptor results in dimerization and autophosphorylation of CSF-1R, triggering signaling cascades that contribute to cytoskeletal remodeling, increases in cell motility and the rate of protein synthesis, and many other outcomes [202, 203]. This cytokine affects a range of macrophage-related immunological functions [201], including the release of IL-10 and IFN-γ, activation of anti-bacterial and anti-fungal activity, and increasing ROS production. M-CSF also plays a role in polarizing resident macrophages into anti-inflammatory type M2 macrophages [204]. Our results show that Cath-2, alongside with LTA, decreased the extracellular concentration of M-CSF, while PMA elevated it. The M-CSF levels were further reduced following the treatment with the lower dose of Cath-2 and LTA compared to the group receiving only LTA, and both concentrations of the peptide decreased the M-CSF levels of the PMA-exposed cells. These results suggest that Cath-2 may suppress macrophage activation, also highlighting the multiplex immunomodulatory role of this AMP.

Though LTA exposure evoked an increase in IFN-γ levels in the hepatocyte- non-parenchymal cell co-cultures, no changes were observed in CXCLi2 and IL-10 concentrations. Similarly, PMA only increased the production of M-CSF from the investigated cytokine/chemokine profile. These results indicate that both LTA and PMA had a pro-inflammatory action on the cells, but this was not reflected by all mediators assessed, probably due to the greatly varied effects of pro-inflammatory molecules in different species and tissue types [199, 205].

Oxidative stress is associated with hepatic inflammatory processes because ROS and RNS are released from hepatocytes, Kupffer cells, and neutrophils in response to PAMPs [206, 207]. Due to the immunomodulatory effects of AMPs, these peptides may play a role in influencing the redox balance as well. Some cathelicidins have been found to act against oxidative stress, but others, however, seem to increase ROS production of immune cells [208, 209]. Several studies found that cathelicidins had a protective effect against lipid peroxidation and the subsequent cell membrane damage [210, 211]. According to our results, Cath-2 dose-dependently increased the production of H_2O_2, but this effect was much less pronounced when applied together with LTA, possibly due to its suggested LTA-binding. Treatment with LTA also elevated the H_2O_2 production of the cultured hepatic cells, an effect which was counteracted by the lower dose of Cath-2, indicating that neither the Cath-2, nor the LTA could perform remarkable pro-oxidant action when applied together. However, these outcomes did not result in an increase in lipid peroxidation, as shown by the unchanged MDA concentrations, which indicates that the increased oxidative load did not lead to enhanced lipid peroxidation, so Cath-2 did not contribute to oxidative damage of cell membranes. Moreover, the higher dose of the peptide decreased the MDA concentration, indicating a protective effect on the integrity of the membrane-forming phospholipids.

Based on our results, it can be stated that Cath-2 plays a substantial role in modulating the hepatic immune response with a multifaceted mode of action. It was found to have dose-dependent effects on metabolic activity, membrane integrity, and ROS production, indicating that using it in excessively high concentrations can lead to cell damage. However, the lower applied dose was not found to elicit any remarkable deteriorative action on cultured liver cells. Our findings give evidence that this molecule can possess anti-inflammatory properties, reflected by the alleviation of the LTA-triggered IFN-γ surge, and as a potent immunomodulator it can also stimulate pro-inflammatory CXCLi2 release balanced by enhanced anti-inflammatory IL-10 production. Further, the complex interplay of endotoxins and AMPs was highlighted as cathelicidin-2 showed less pronounced effects in the presence of LTA due to its binding capability, also neutralizing the endotoxin-associated inflammatory response. In conclusion, cathelicidin-2 seems to be a promising candidate for future immunomodulating drug development with an attempt to reduce the application of antibiotics.

7.3 Study II: Effects of IDR-1002 on a primary hepatic cell culture

IDRs gained attention in recent years through their ability to protect against bacterial infections without direct antimicrobial effect. They seem to be able to modulate the host immune response by enhancing chemokine production and leukocyte recruitment, while being able to suppress harmful inflammation [212]. In this study it was intended to examine the effects of one peptide of the IDR family, IDR-1002 on the hepatic immune response of the chicken by measuring seven cytokines/chemokines (IL-6, CXCLi2, IFN-γ, IL-16, IL-10, M-CSF and RANTES), while testing whether they have a negative effect on cell viability.

Previous research suggests that, unlike cathelicidins, IDRs need much higher concentrations in order to be cytotoxic [52, 213, 214]. In the present study, IDR-1002 was examined in concentrations of 10, 30 and 90 µg/mL and did not alter the metabolic activity of the cells. There was a significant, moderate increase in extracellular LDH activity after treatment of the cells with both LTA and the highest concentration of the peptide together, suggesting some degree of membrane damage. This indicates that, although the cell-damaging effects of the peptide are generally negligible, it would still worth selecting the lowest effective concentration for therapeutic use.

One of the main aims of this experiment was to determine how the primary immune cells of the liver, Kupffer cells, respond to treatment with the peptide. For this purpose, such cytokines were investigated that have a strong effect on macrophage differentiation. Firstly, the level of RANTES was measured in our experiment, and it was found that treatment with high concentrations of IDR-1002 (90 µg/mL) and LTA increased the concentration of this protein, and cell cultures treated with both IDR-1002 and LTA showed an even more pronounced concentration-dependent increase. RANTES, also known as CCL5 (C-C motif ligand 5), is a chemokine produced in the liver by hepatocytes, macrophage stellate cells and endothelial cells. It stimulates leukocyte migration, modulates the production of several cytokines and has a complex role in the regulation of inflammatory processes [215]. Other studies also shown similar results to ours, for example showing the increase of RANTES levels in a murine infection model after treatment with IDR-1002 [86], furthermore, IDR-1 was shown to stimulate signaling pathways contributing to the induction of RANTES [52]. In addition, IDR-1002 was found to activate the migration of monocytes towards RANTES, thereby stimulating bacterial elimination [216].

RANTES was shown to promote the conversion of macrophages to the M1 form (pro-inflammatory) and inhibit the M2 form (anti-inflammatory) [215]. In contrast, according to our findings, M-CSF levels were also increased after IDR-1002 treatment and were unchanged by

LTA alone. M-CSF is produced by hepatocytes and Kupffer cells in the liver and plays an important role in the differentiation of Kupffer cells into M2-type macrophages and in the regulation of regenerative processes [217, 218]. This seemingly contradictory result is in line with research that found, based on gene expression studies, that IDR-1018 promoted a phenotypic transition between the two forms [87], while e.g. LL-37 stimulated M1 macrophage formation [219]. Both types of macrophages play an important role in inflammatory processes. M2 macrophages are able to perform active phagocytosis, produce high levels of chemotactic factors and IL-10, therefore are usually referred to as anti-inflammatory cells [220]. M1 macrophages, in contrast, produce proinflammatory cytokines, have anti-tumour immune promoting activity, but can also play a role in tissue damage [56].

Although the fact that the levels of certain pro-inflammatory cytokines decreased in our case following IDR-1002 treatment (IFN-γ, IL-6, IL-16) would suggest that macrophages preferentially recruited the anti-inflammatory form promoted by M-CSF, in somewhat contrast to this, we found that IL-10 levels also decreased following treatment with the peptide (and were not affected by LTA), which may rather indicate a predominance of a process driven by RANTES. This is further confirmed by the strong positive correlations which were found between RANTES and IFN-γ, between RANTES and IL-16, and interestingly, between RANTES and IL-10 (see **Supplemetary information file 1**). In addition, there was a negative correlation between M-CSF and CXCLi2, and a mentionable, but not significant positive correlation between M-CSF and IL-10. This supports the idea that macrophages stimulated by IDR-1002 drive a transition state between the two phenotypes.

As mentioned above, the decrease of IL-6 and IFN-γ concentrations was observed after treatment with IDR-1002. Notwithstanding that IDR-1002 was shown to increase the production of these chemokines in various studies [86, 221, 222], the effect of the peptide on different immune cells is not fully understood, and to the best of our knowledge, no research has been published on its action on hepatocytes and Kupffer cells. In the work of Huante-Mendoza *et. al* [223] it was found that the translocation of NF-κB into the nucleus and the CREB (cAMP-response element binding protein)-CBP (CREB binding protein) complex formation was inhibited by the peptide after LPS treatment, suggesting that the induction of pro- and anti-inflammatory chemokines (such as IL-6, IL-8 or IL-10) was obstructed. Further, IDR-1002 suppressed the inflammation induced by *Pseudomonas aeruginosa* infection in mouse lung, and did not enhance the production of the tested cytokines including IL-6 and TNF-α [221].

As mentioned before, IFN-γ is known to activate macrophages and promote the pro-inflammatory-, while suppressing the anti-inflammatory cytokine production [224]. The production of IFN-γ by Kupffer cells can also lead to acute liver damage, or worsen the existing

chronic hepatic failure [225, 226]. Following LTA treatment, IFN-γ levels were elevated, indicating the pro-inflammatory effect of the applied endotoxin, which was reduced by IDR-1002 treatment at all three concentrations, suggesting that the peptide was successful in alleviating the inflammatory response.

IDR-1002 did not significantly affect the levels of CXCLi2 (corresponding to IL-8 in chickens) when solely applied in our experiments, but the concentration of this cytokine was increased by LTA treatment, which was reduced by 30 and 90 µg/mL concentrations of the peptide. Similar results were shown in a previous study where IDR-1 successfully reduced LPS-induced IL-8 activation [52], and also where IDR-1002 dampened IL-1β-induced IL-8 production [227]. Similarly to these results, LTA-induced IL-6 levels were also diminished by IDR-1002, but in this case, treatment with the peptide alone also decreased the level of this interleukin. Previous studies showed that IDR-1002 was able to reduce IL-6 production in a *Pseudomonas aeruginosa* lung infection model [221, 222], and in a *Staphylococcus aureus* treated RAW 264.7 macrophage cell line [89].

In the present study, IL-16 levels were decreased in cells co-treated with LTA and IDR-1002. IL-16 is a chemokine that stimulates the migration of CD14+ immune cells and may cause tissue damage mediated by pro-inflammatory processes and plays an important role in mediating liver cell apoptosis. It can be seen that its reduced levels may have several advantages in the event of an inflammatory response [228, 229].

Phagocytosis by various pathogenic microorganisms results in the formation of lysosomes, which contain high amounts of ROS and RNS [230]. The associated oxidative stress helps to eliminate pathogens, but their excessive production can cause tissue damage, and they have also been shown to play a role in acute liver injury [231–233]. Similarly to the pro-inflammatory cytokines IL-6 and IFN-γ, IDR-1002 also reduced the level of H_2O_2 in the medium (at a concentration of 30 µg/mL), which may also contribute to the reduction of cell damage.

The IDR-1002-driven decrease in ROS level could be explained by the elevated abundance of the transcription factor Nrf2 in IDR-1002 treated cells. The signaling pathway regulated by Nrf2/KEAP1 (nuclear factor erythroid 2-related factor 2/ Kelch-like ECH-associated protein 1) plays an important role in protecting cells from oxidative stress. Nrf2 is degraded in the proteosomes in reaction with KEAP, but in the presence of ROS, RNS or certain protective molecules (e.g. isothiocyanates, organosulfur compounds, polyphenols), the cysteine residues of KEAP1 are oxidized, leading to the dissociation of Nrf2 and its ability to enter the nucleus where it binds to various AREs (antioxidant responsive elements), leading to the activation of numerous antioxidant systems (e.g. superoxide dismutase, glutathione peroxidase) [232, 234]. In addition, LTA also increased Nrf2 levels, which, may have been caused by a compensatory response, as Nrf2 also plays a role in the regulation of innate

immunity, providing protection against excessive inflammatory processes. It has been shown that ROS levels elevated by LTA or LPS activated the translocation of Nrf2, triggering the cellular defense system [235, 236].

Interestingly, carbonylated protein levels were elevated by all three concentrations of IDR-1002. This outcome could be connected to the fact that some AMPs (including IDR-1002) proved to have potent antibiofilm effect [237–239]. As biofilms contain large amount of proteins [240], it can be beneficial for the peptide to be able to carbonylate them. For example, the AMP KWI18 was found to inhibit biofilm formation while inducing lipid-, and protein oxidation [241]. In our case, protein carbonyl formation hasn't been linked with ROS production, and did not cause any damage to the cells. Based on these results, the role of IDR-1002 in oxidative stress may be worth further investigation by including other parameters.

In conclusion, it can be stated that IDR-1002 has a complex effect on the regulation of the immune system of the chicken liver. It showed to reduce the LTA-induced pro-inflammatory response (as seen for IFN-γ, IL-6, CXCLi2 and IL-16), suggesting that it may be a promising candidate for attenuating pathological inflammatory processes. Moreover, the measured parameters suggested that macrophage activity was modulated to an intermediate state between anti- and pro-inflammatory forms, as IDR-1002 reduced both the concentration of pro-inflammatory cytokines (IFN-γ, IL-6) and the anti-inflammatory IL-10. This effect can be well explained by the increase in the level of RANTES (redirecting macrophages towards the pro-inflammatory M1 type) and M-CSF (stimulating differentiation towards the anti-inflammatory M2 type). In addition, we can conclude that IDR-1002 can also affect the oxidative status of cells by reducing the levels of extracellular H_2O_2, presumably due to its inductive effect on the Nrf2 signaling pathway. It was also able to attenuate LTA-induced H_2O_2 production accompanied by Nrf2 induction. Thus, it can be stated that IDR-1002 has a highly complex effect on the cellular immune response and, although further extensive studies are undoubtedly needed to elucidate its exact mechanism, it may be a promising candidate for the treatment of pathologies associated with inflammation of bacterial origin.

7.4 Study III: Effects of Cath-2 on an intestinal explant culture

First, it was aimed to examine the potential cytotoxic effects of Cath-2 on the intestinal explant cultures. As previously described, the peptide can negatively influence the viability of the hepatic cell cultures when used in higher concentrations. This could be either explained by the sensitivity of the primary isolated cells, or by the possibility that liver cells can be inherently more reactive when contacting foreign substances.

Nonetheless, this effect did not occur with the chicken intestinal explants used in this study, as neither the metabolic activity, nor the LDH activity was altered by 5 and 10 nmol/mL concentrations of Cath-2 which might imply the applicability of Cath-2 for gastrointestinal diseases. Although, it is worth considering, that the highest concentration of Cath-2 treatment (25 nmol/mL) significantly increased the metabolic activity of the cells, by approximately 200%.

The increased metabolic activity can not only be caused by the production of $NADH+H^+$, as the CCK-8 test utilized in this study also measures the cellular production of $NADPH+H^+$, which is known to have a diverse role in the regulation of inflammatory mechanisms [242]. For example, $NAPDH+H^+$ is required for fatty acid synthesis, the upregulation of which is characteristic of inflammatory processes. Fatty acids are necessary to produce pro-inflammatory lipid mediators (like prostaglandin E_2) [243] and have an important role in the organization of lipid rafts [244], and in the regulation of phagocytosis due to the need of membrane formation [245, 246]. The Cath-2-associated increased production of $NAPDH+H^+$ aligns with our results indicating the pro-inflammatory effects of the same concentration of Cath-2 as it elevated the IL-2 and CXCLi2 levels as well.

Activation of the immune response to enhance antimicrobial activity is an important function of cathelicidins and has been established by several previous studies. For example, it has been proven that the human cathelicidin LL-37, as well as Cath-2, activates the NLRP-3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome, which leads to the caspase-1-dependent release of pro-inflammatory cytokines [247, 248]. In the present study, the sole application of Cath-2 increased the levels of CXCLi2, IL-6 and IL-2, and the cellular CXCLi2 release was stimulated by 25 nmol/mL Cath-2, even under LTA-provoked inflammation. These findings are in line with other results obtained from the research of Cath-2 (and Cath-2 derived peptides), where it has been able to induce the production of different cytokines and chemokines (e.g. IL-8, RANTES, IL-1β and IL-1α) [72, 184, 248, 249].

IL-2 has an especially important role in gut homeostasis as it enhances immune tolerance and prevents chronic inflammation of the intestinal mucosa by adjusting the function of regulatory T-cells [250–253]. It is mainly produced by activated T-cells during inflammation and has both pro- and anti-inflammatory functions [254]. In this study, both, Cath-2 and LTA applied alone elevated the levels of this cytokine, while Cath-2 lowered the LTA-caused increase, indicating a protective and anti-inflammatory effect of this AMP. However, to further evaluate the protective function of Cath-2 in the immune system of the intestines, additional research is needed regarding the distribution of different T-cell populations, the lack of which is a limitation of this study.

The pro-inflammatory effects of LTA on chicken intestinal explant cultures and chicken hepatic cell cultures have been investigated previously by our research group, where LTA was shown to be a suitable tool to stimulate inflammatory responses [162]. Based on our present

results, Cath-2 had mostly anti-inflammatory effects under LTA-evoked inflammatory conditions as it successfully alleviated the LTA-triggered elevation of IL-2 and IL-6 levels, as well as IFN-γ/IL-10 ratio. Given their essential roles in regulating inflammatory processes, particularly in the intestinal mucosa where IFN-γ acts as a pro-inflammatory cytokine and IL-10 as an anti-inflammatory counterpart, this ratio provided valuable insights into the dynamics of the inflammatory state [255–258]. Similar results were obtained from previous studies where the anti-inflammatory properties of this peptide and its derivates were investigated. For example, they have been proven to neutralize the LPS and LTA-induced release of pro-inflammatory cytokines [77, 78, 184] and to inhibit *Pseudomonas aeruginosa*-caused macrophage activation [259].

Furthermore, in this study, LTA decreased the concentration of the anti-inflammatory IL-10, and Cath-2 raised it back to the level observed in the Control group. Moreover, the production of IFN-γ was elevated after treatment with LTA, but Cath-2 did not affect its level. Despite this, the LTA-caused elevation in the IFN-γ/IL-10 ratio was lowered by all concentrations of this AMP, suggesting an explicit anti-inflammatory mechanism.

IL-10 is an immunomodulatory cytokine, with an especially important anti-inflammatory role in the intestines. It is produced by a wide range of cell types (including regulatory T cells) and acts mainly on lymphocytes and myeloid cells to suppress numerous pathways of the immune response [246]. In contrast, IFN-γ is considered pro-inflammatory: it is produced mainly by T-helper cells and Natural Killer (NK) cells and plays a crucial role in immune cell activation, antimicrobial immune responses, antigen presentation, cellular proliferation, and apoptosis [247]. The ratio of these two cytokines is especially useful in studies involving the intestinal immune system as they both play a particularly important role in its regulation [255–257, 260, 261].

In the recent study, the anti-inflammatory effects of Cath-2 were the most prominent at 25 nmol/mL concentration as it was the only one decreasing IL-2 and IL-6 concentrations, the IFN-γ/IL-10 ratio, and elevating the IL-10 levels compared to the group receiving only LTA.

In conclusion, our present results provide important novel data on the immunomodulatory effects of Cath-2 on the small intestinal mucosa of the chicken. Based on the data gained, it can be stated that Cath-2 has a broad anti-inflammatory effect on chicken intestinal explants as reflected by the alleviation of the LTA-triggered pro-inflammatory cytokine release, mostly at higher concentrations, without being cytotoxic. Moreover, the immunostimulant action of this AMP could also be observed, predominantly when administered to non-inflamed cells, which can prove to be an efficient mechanism to provide protection against infections. Therefore, Cath-2 can be considered a suitable candidate for the treatment of bacterial diseases by maintaining the appropriate inflammatory homeostasis and with further

in vivo studies may significantly contribute to the reduction of antibiotic use for enteric infections.

7.5 Study IV: Effects of IDR-1002 on an intestinal explant culture

In this study, it was again demonstrated that the IDR-1002 peptide does not have a cytotoxic effect if the right concentration is chosen. The metabolic activity of the chicken intestinal explants employed in this experiment was not altered, confirming the absence of cytotoxicity caused by IDR-1002, while the extracellular LDH activity was decreased after treatment with 30 µg/mL IDR-1002. One possible explanation for this phenomenon could be the cytoprotective autophagy, which has been described to cause decreased LDH levels previously [262–264]. Conversely, this concentration of the peptide similarly elevated the CXCLi2 and IL-2 levels, indicating a pro-inflammatory mechanism that may eventuate in the cells entering a protective state. However, it is important to note that clarifying the reason for the observed lower LDH release is not possible in our study, which can be considered as a limitation.

AMPs have exceedingly diverse mechanisms of action. One of them is the stimulation of the production of various cytokines and chemokines to enhance the intrinsic defense against pathogenic microbes [86]. This effect of the IDR-1002 peptide has been reported previously in different cell types and *in vivo* studies as well [86, 222]. Regarding our data, mainly the 30 µg/mL concentration of the peptide had this impact, as only this elevated the CXCLi2 and IL-2 concentrations at the same time, however, all concentrations increased the IL-2 production of the explants. Our results align with the work of Kuerth et al., where the peptide stimulated the IL-8 production of human bronchial epithelial cells [222].

As mentioned before, IL-2 has a crucial role in the intestinal mucosa, which includes the promotion of immune tolerance and immune memory, and the prevention of chronic inflammation by modulating the function of T-regulatory cells [250–252]. For example, in a mouse model, it was shown to be essential for maintaining the immune homeostasis of the small intestine. Without its production, the tolerance to dietary antigens becomes severely disabled [253]. In this study, similarly to the case of Cath-2, the production of IL-2 was enhanced after treatment of IDR-1002 and after LTA, yet the LTA-caused elevation was decreased after the addition of 10 µg/mL IDR-1002 treatment. Regarding this data, IDR-1002 may enhance the self-protective abilities of the intestinal mucosa and provide defense against chronic inflammatory processes, however, further studies are needed to confirm this hypothesis.

In this study, LTA elevated the concentrations of IFN-γ, CXCLi2, IL-2, RANTES, and also the ratio of IFN-γ/IL-10, indicating a successful attempt to induce inflammation in the chicken intestinal explant model. These results align with the ones gained from Study III, and also with a preliminary study conducted by our research group [162] where LTA also showed a pro-inflammatory effect on the intestinal explants.

The anti-inflammatory properties of IDR-1002 have been stated by several previous studies [84, 89, 222, 223, 227, 265]. The LTA-induced inflammation in our study was diminished by IDR-1002, although, there were differences among the three concentrations. The lowest, 30 μg/mL application seemed to be the most potent, as it was the only one to decrease CXCLi2, IL-2, and RANTES levels (the latter is known to induce macrophage differentiation into the pro-inflammatory M1 form [215]). Meanwhile, the highest, 90 μg/mL concentration decreased the IFN-γ/IL-10 ratio (but elevated IFN-γ production), strongly increased the anti-inflammatory IL-10, and decreased CXCLi2 levels.

The ratio of the two cytokines, IFN-γ and IL-10 is used especially in research related to the intestinal immune system, and in other areas as well [255–258]. Although the highest concentration of IDR-1002 elevated the levels of IFN-γ, the ratio was lowered as a consequence of the intensely increased IL-10 production of the explants – which, considering the literature data, means that the overall pro-inflammatory state of the cells was taken under control by the production of IL-10. The same concentration of the peptide raised IL-2 levels when applied alone and did not decrease the LTA-induced elevation.

In conclusion, while IDR-1002 did not possess any negative effect on the viability of the explant models, it had a major impact on inflammatory processes. Our results propose that there are significant differences between the three concentrations considered here, suggesting that the effect of the peptide is not negligibly dependent on the dose used. With this in mind, further studies are certainly needed to explore the differences between the different concentrations and to select the most optimal one. Nevertheless, it can be stated that the IDR-1002 AMP presented a potent immunomodulatory action on intestinal explants. The peptide alone enhanced the innate defense mechanisms by stimulating IL-2 and CXCLi2 production, while it alleviated the LTA-evoked pro-inflammatory cytokine peak (reflected by IL-2, CXCLi2, RANTES concentrations, and by the IFN-γ/IL-10 ratio), thus restoring the physiological inflammatory homeostasis. These data suggest that IDR-1002 might be a promising candidate in the multimodal treatment of inflammation caused by bacterial infections of intestinal origin in chicken.

7.6 Conclusion

Our five studies demonstrate the diversified effects of AMPs, while showing their dependence on different biological factors. Therefore, two AMPs might influence the immune response of the same cell type in somewhat different ways, while one AMP might provoke diverse immune responses in different tissues or organs. Consequently, it can be concluded that a more substantial understanding of the cellular effects of AMPs is necessary before they can be introduced into clinical research. Our results can contribute to this goal considerably by providing valuable data on cell viability and immune response provoked by two important peptides, the Cath-2 and IDR-1002 in two *in vitro* model systems.

As the resistance of infectious agents to the available chemotherapic drugs is getting increasingly serious, new ways have to be developed to combat these diseases. AMPs might provide an option to augment the use of antibiotics due to their wide range of effects on the immune system. AMPs show that utilizing the organism's own immune system to achieve this goal is an interesting approach in this search. While using the innate competence of the immune system to provide a possible noble solution to kill pathogenic bacteria, they have a wide range of abilities to combat harmful inflammation and to help the body regenerate more swiftly.

We hope that studies such as ours can provide invaluable basis for future research in this interesting field.

8 New scientific results

Ad 1,

A reliable inflammatory model has been successfully established for the hepatocyte – non-parenchymal cell co-culture framework. LTA and, to a lesser extent, PMA has been proven to effectively induce inflammation and are suitable for the testing of immunomodulatory materials using our model.

Ad 2,

Chicken cathelicidin-2 decreased cell viability in the hepatocyte – non-parenchymal cell co-culture model, especially when used in higher than 5 nmol/mL concentrations. The viability of the intestinal explants was not reduced by Cath-2. In contrast, IDR-1002 did not have an effect on cell viability of either the hepatocyte – non-parenchymal cell co-culture model or the intestinal explants.

Ad 3,

Chicken cathelicidin-2 had a diversified role in the modulation of the immune response of the hepatocyte – non-parenchymal cell co-culture model and the intestinal explants. It had a pro-inflammatory effect in both models when applied alone, especially at higher concentrations. However, it also demonstrated anti-inflammatory effects as it alleviated inflammation provoked by LTA in both models.

Ad 4,

IDR-1002 had a complex role in modulating the immune response of the hepatocyte – non-parenchymal cell co-culture model and the intestinal explants. It had a remarkable anti-inflammatory effect as it decreased the pro-inflammatory cytokine release induced by LTA in both models. Meanwhile, based on the RANTES, M-CSF and cytokine concentrations, it steered inflammatory processes into an intermediate phase between the anti- and proinflammatory direction when used on the hepatocyte – non-parenchymal cell co-culture model.

9 References

1. Jago W, Jago W (1926) Toxic action of wheat flour to brewer's yeast. Ind Ferment 128–167

2. Balls AK, Hale WS, Harris TH (1942) A crystalline protein obtained from a lipoprotein of wheat flour. Cereal Chem 19:279–288

3. Stec B (2006) Plant thionins – the structural perspective. Cell Mol Life Sci CMLS 63:1370–1385. https://doi.org/10.1007/s00018-005-5574-5

4. Bennett JW, Chung K-T (2001) Alexander Fleming and the discovery of penicillin. In: Advances in Applied Microbiology. Academic Press, pp 163–184

5. Dubos RJ (1939) Studies on a bactericidal agent extracted from a soil bacillus. J Exp Med 70:1–10

6. Hotchkiss RD, Dubos RJ (1940) Chemical properties of bactericidal substances isolated from cultures of a soil bacillus. J Biol Chem 132:793–794

7. Van Epps HL (2006) René Dubos: unearthing antibiotics. J Exp Med 203:259. https://doi.org/10.1084/jem.2032fta

8. Dubos RJ, Hotchkiss RD (1941) The production of bactericidal substances by aerobic sporulating Bacilli. J Exp Med 73:629–640. https://doi.org/10.1084/jem.73.5.629

9. Uddin TM, Chakraborty AJ, Khusro A, Zidan BRM, Mitra S, Emran TB, Dhama K, Ripon MdKH, Gajdács M, Sahibzada MUK, Hossain MdJ, Koirala N (2021) Antibiotic resistance in microbes: History, mechanisms, therapeutic strategies and future prospects. J Infect Public Health 14:1750–1766. https://doi.org/10.1016/j.jiph.2021.10.020

10. Nakatsuji T, Gallo RL (2012) Antimicrobial Peptides: Old Molecules with New Ideas. J Invest Dermatol 132:887–895. https://doi.org/10.1038/jid.2011.387

11. Hirsch JG (1956) Phagocytin: A bactericidal substance from polymorphonuclear leucocytes. J Exp Med 103:589–611

12. Kiss G, Michl H (1962) Uber das Giftsekret der Gelbbauchunke, Bombina variegata L. Toxicon 1:33–34. https://doi.org/10.1016/0041-0101(62)90006-5

13. Ageitos JM, Sánchez-Pérez A, Calo-Mata P, Villa TG (2017) Antimicrobial peptides (AMPs): Ancient compounds that represent novel weapons in the fight against bacteria. Biochem Pharmacol 133:117–138. https://doi.org/10.1016/j.bcp.2016.09.018

14. Sorensen M, Sorensen SPL (1940) The proteins in whey. C r Trav Lab Carlsberg Ser Chim 23:55–99

15. Groves ML (1960) The Isolation of a Red Protein from Milk. ACS Publ. https://doi.org/10.1021/ja01498a029

16. Johanson B (1960) Isolation of an iron-containing red protein from milk. Acta Chem Scand 14:510–512

17. Montreuil J, Tonnelat J, Mullet S (1960) Preparation and properties of the lactosiderophilin (lactotransferrine) of human milk. Biochim Biophys Acta 45:413–21

18. Kirkpatrick CH, Green I, Rich RR, Schade AL (1971) Inhibition of Growth of Candida albicans by Iron-Unsaturated Lactoferrin: Relation to Host-Defense Mechanisms in Chronic Mucocutaneous Candidiasis. J Infect Dis 124:539–544. https://doi.org/10.1093/infdis/124.6.539

19. Stephens JM, Marshall JH (1962) Some properties of an immune factor isolated from the blood of actively immunized wax moth larvae. Can J Microbiol 8:719–725. https://doi.org/10.1139/m62-093

20. Levy O (2004) Antimicrobial proteins and peptides: anti-infective molecules of mammalian leukocytes. J Leukoc Biol 76:909–925. https://doi.org/10.1189/jlb.0604320

21. Arnold RR, Cole MF, McGhee JR (1977) A Bactericidal Effect for Human Lactoferrin. Science 197:263–265. https://doi.org/10.1126/science.327545

22. Weiss J, Elsbach P, Olsson I, Odeberg H (1978) Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes. J Biol Chem 253:2664–2672

23. Selsted ME, Brown DM, DeLange RJ, Lehrer RI (1983) Primary structures of MCP-1 and MCP-2, natural peptide antibiotics of rabbit lung macrophages. J Biol Chem 258:14485–14489. https://doi.org/10.1016/S0021-9258(17)43888-9

24. Gabay JE, Scott RW, Campanelli D, Griffith J, Wilde C, Marra MN, Seeger M, Nathan CF (1989) Antibiotic proteins of human polymorphonuclear leukocytes. Proc Natl Acad Sci 86:5610–5614. https://doi.org/10.1073/pnas.86.14.5610

25. Hultmark D, Steiner H, Rasmuson T, Boman HG (1980) Insect Immunity. Purification and Properties of Three Inducible Bactericidal Proteins from Hemolymph of Immunized Pupae of Hyalophora cecropia. Eur J Biochem 106:7–16. https://doi.org/10.1111/j.1432-1033.1980.tb05991.x

26. Steiner H, Hultmark D, Engström Å, Bennich H, Boman HG (1981) Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature 292:246–248. https://doi.org/10.1038/292246a0

27. Zasloff M (1987) Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc Natl Acad Sci 84:5449–5453. https://doi.org/10.1073/pnas.84.15.5449

28. Ganz T (2003) The Role of Antimicrobial Peptides in Innate Immunity1. Integr Comp Biol 43:300–304. https://doi.org/10.1093/icb/43.2.300

29. Lemaitre B, Nicolas E, Michaut L, Reichhart J-M, Hoffmann JA (1996) The Dorsoventral Regulatory Gene Cassette spätzle/Toll/cactus Controls the Potent Antifungal Response in Drosophila Adults. Cell 86:973–983. https://doi.org/10.1016/S0092-8674(00)80172-5

30. Thomas P, Vineeth Kumar TV, Reshmy V, Kumar KS, George S (2012) A mini review on the antimicrobial peptides isolated from the genus Hylarana (Amphibia: Anura) with a proposed nomenclature for amphibian skin peptides. Mol Biol Rep 39:6943–6947. https://doi.org/10.1007/s11033-012-1521-3

31. Nicolas P, El Amri C (2009) The dermaseptin superfamily: A gene-based combinatorial library of antimicrobial peptides. Biochim Biophys Acta BBA - Biomembr 1788:1537–1550. https://doi.org/10.1016/j.bbamem.2008.09.006

32. Fernandez DI, Gehman JD, Separovic F (2009) Membrane interactions of antimicrobial peptides from Australian frogs. Biochim Biophys Acta BBA - Biomembr 1788:1630–1638. https://doi.org/10.1016/j.bbamem.2008.10.007

33. Barbosa Pelegrini P, del Sarto RP, Silva ON, Franco OL, Grossi-de-Sa MF (2011) Antibacterial Peptides from Plants: What They Are and How They Probably Work. Biochem Res Int 2011:e250349. https://doi.org/10.1155/2011/250349

34. Yin LM, Edwards MA, Li J, Yip CM, Deber CM (2012) Roles of Hydrophobicity and Charge Distribution of Cationic Antimicrobial Peptides in Peptide-Membrane Interactions *. J Biol Chem 287:7738–7745. https://doi.org/10.1074/jbc.M111.303602

35. Kumar P, Kizhakkedathu JN, Straus SK (2018) Antimicrobial Peptides: Diversity, Mechanism of Action and Strategies to Improve the Activity and Biocompatibility In Vivo. Biomolecules 8:4. https://doi.org/10.3390/biom8010004

36. Guaní-Guerra E, Santos-Mendoza T, Lugo-Reyes SO, Terán LM (2010) Antimicrobial peptides: general overview and clinical implications in human health and disease. Clin Immunol Orlando Fla 135:1–11. https://doi.org/10.1016/j.clim.2009.12.004

37. Brogden KA (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3:238–250. https://doi.org/10.1038/nrmicro1098

38. Shai Y (2002) Mode of action of membrane active antimicrobial peptides. Pept Sci 66:236–248. https://doi.org/10.1002/bip.10260

39. Ehrenstein G, Lecar H (1977) Electrically gated ionic channels in lipid bilayers. Q Rev Biophys 10:1–34. https://doi.org/10.1017/S0033583500000123

40. Wimley WC (2010) Describing the Mechanism of Antimicrobial Peptide Action with the Interfacial Activity Model. ACS Chem Biol 5:905–917. https://doi.org/10.1021/cb1001558

41. Yeaman MR, Yount NY (2003) Mechanisms of Antimicrobial Peptide Action and Resistance. Pharmacol Rev 55:27–55. https://doi.org/10.1124/pr.55.1.2

42. Wu M, Maier E, Benz R, Hancock RE (1999) Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of Escherichia coli. Biochemistry 38:7235–7242. https://doi.org/10.1021/bi9826299

43. Zhang L, Benz R, Hancock REW (1999) Influence of Proline Residues on the Antibacterial and Synergistic Activities of α-Helical Peptides. Biochemistry 38:8102–8111. https://doi.org/10.1021/bi9904104

44. Graf M, Wilson DN (2019) Intracellular Antimicrobial Peptides Targeting the Protein Synthesis Machinery. In: Matsuzaki K (ed) Antimicrobial Peptides: Basics for Clinical Application. Springer, Singapore, pp 73–89

45. Park CB, Kim HS, Kim SC (1998) Mechanism of Action of the Antimicrobial Peptide Buforin II: Buforin II Kills Microorganisms by Penetrating the Cell Membrane and

Inhibiting Cellular Functions. Biochem Biophys Res Commun 244:253–257. https://doi.org/10.1006/bbrc.1998.8159

46. Malanovic N, Lohner K (2016) Antimicrobial Peptides Targeting Gram-Positive Bacteria. Pharmaceuticals 9:59. https://doi.org/10.3390/ph9030059

47. Hancock REW, Scott MG (2000) The role of antimicrobial peptides in animal defenses. Proc Natl Acad Sci 97:8856–8861. https://doi.org/10.1073/pnas.97.16.8856

48. Gallo RL, Hooper LV (2012) Epithelial antimicrobial defence of the skin and intestine. Nat Rev Immunol 12:503–516. https://doi.org/10.1038/nri3228

49. Ganz T (1987) Extracellular release of antimicrobial defensins by human polymorphonuclear leukocytes. Infect Immun 55:568–571. https://doi.org/10.1128/iai.55.3.568-571.1987

50. Teltschik Z, Wiest R, Beisner J, Nuding S, Hofmann C, Schoelmerich J, Bevins CL, Stange EF, Wehkamp J (2012) Intestinal bacterial translocation in rats with cirrhosis is related to compromised paneth cell antimicrobial host defense. Hepatology 55:1154–1163. https://doi.org/10.1002/hep.24789

51. Hancock REW, Haney EF, Gill EE (2016) The immunology of host defence peptides: beyond antimicrobial activity. Nat Rev Immunol 16:321–334. https://doi.org/10.1038/nri.2016.29

52. Scott MG, Dullaghan E, Mookherjee N, Glavas N, Waldbrook M, Thompson A, Wang A, Lee K, Doria S, Hamill P, Yu JJ, Li Y, Donini O, Guarna MM, Finlay BB, North JR, Hancock REW (2007) An anti-infective peptide that selectively modulates the innate immune response. Nat Biotechnol 25:465–472. https://doi.org/10.1038/nbt1288

53. Hilchie AL, Wuerth K, Hancock REW (2013) Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. Nat Chem Biol 9:761–768. https://doi.org/10.1038/nchembio.1393

54. van der Does AM, Hiemstra PS, Mookherjee N (2019) Antimicrobial Host Defence Peptides: Immunomodulatory Functions and Translational Prospects. In: Matsuzaki K (ed) Antimicrobial Peptides: Basics for Clinical Application. Springer, Singapore, pp 149–171

55. Van Wetering S, Mannesse-Lazeroms SP, Van Sterkenburg MA, Daha MR, Dijkman JH, Hiemstra PS (1997) Effect of defensins on interleukin-8 synthesis in airway epithelial cells. Am J Physiol 272:L888-896. https://doi.org/10.1152/ajplung.1997.272.5.L888

56. Yunna C, Mengru H, Lei W, Weidong C (2020) Macrophage M1/M2 polarization. Eur J Pharmacol 877:173090. https://doi.org/10.1016/j.ejphar.2020.173090

57. van der Does AM, Beekhuizen H, Ravensbergen B, Vos T, Ottenhoff THM, van Dissel JT, Drijfhout JW, Hiemstra PS, Nibbering PH (2010) LL-37 directs macrophage differentiation toward macrophages with a proinflammatory signature. J Immunol Baltim Md 1950 185:1442–1449. https://doi.org/10.4049/jimmunol.1000376

58. Lee MO, Jang H-J, Rengaraj D, Yang S-Y, Han JY, Lamont SJ, Womack JE (2016) Tissue expression and antibacterial activity of host defense peptides in chicken. BMC Vet Res 12:231. https://doi.org/10.1186/s12917-016-0866-6

59. Vandamme D, Landuyt B, Luyten W, Schoofs L (2012) A comprehensive summary of LL-37, the factotum human cathelicidin peptide. Cell Immunol 280:22–35. https://doi.org/10.1016/j.cellimm.2012.11.009

60. Nijnik A, Hancock REW (2009) The roles of cathelicidin LL-37 in immune defences and novel clinical applications. Curr Opin Hematol 16:41–47. https://doi.org/10.1097/moh.0b013e32831ac517

61. Mily A, Rekha RS, Kamal SMM, Arifuzzaman ASM, Rahim Z, Khan L, Haq MA, Zaman K, Bergman P, Brighenti S, Gudmundsson GH, Agerberth B, Raqib R (2015) Significant Effects of Oral Phenylbutyrate and Vitamin D3 Adjunctive Therapy in Pulmonary Tuberculosis: A Randomized Controlled Trial. PLOS ONE 10:e0138340. https://doi.org/10.1371/journal.pone.0138340

62. Mookherjee N, Hamill P, Gardy J, Blimkie D, Falsafi R, Chikatamarla A, Arenillas DJ, Doria S, Kollmann TR, Hancock REW (2009) Systems biology evaluation of immune responses induced by human host defence peptide LL-37 in mononuclear cells. Mol Biosyst 5:483–496. https://doi.org/10.1039/b813787k

63. Mookherjee N, Brown KL, Bowdish DME, Doria S, Falsafi R, Hokamp K, Roche FM, Mu R, Doho GH, Pistolic J, Powers J-P, Bryan J, Brinkman FSL, Hancock REW (2006) Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. J Immunol Baltim Md 1950 176:2455–2464. https://doi.org/10.4049/jimmunol.176.4.2455

64. Benincasa M, Skerlavaj B, Gennaro R, Pellegrini A, Zanetti M (2003) In vitro and in vivo antimicrobial activity of two alpha-helical cathelicidin peptides and of their synthetic analogs. Peptides 24:1723–1731. https://doi.org/10.1016/j.peptides.2003.07.025

65. Veldhuizen EJA, Brouwer EC, Schneider VAF, Fluit AC (2013) Chicken Cathelicidins Display Antimicrobial Activity against Multiresistant Bacteria without Inducing Strong Resistance. PLOS ONE 8:e61964. https://doi.org/10.1371/journal.pone.0061964

66. Schneider VAF, Coorens M, Ordonez SR, Tjeerdsma-van Bokhoven JLM, Posthuma G, van Dijk A, Haagsman HP, Veldhuizen EJA (2016) Imaging the antimicrobial mechanism(s) of cathelicidin-2. Sci Rep 6:32948. https://doi.org/10.1038/srep32948

67. Palacios JB, Barrios-Payán J, Mata-Espinosa D, Lara-Espinosa JV, León-Contreras JC, Lushington GH, Melgarejo T, Hernández-Pando R (2022) In Vitro, In Vivo and In Silico Assessment of the Antimicrobial and Immunomodulatory Effects of a Water Buffalo Cathelicidin (WBCATH) in Experimental Pulmonary Tuberculosis. Antibiot Basel Switz 12:75. https://doi.org/10.3390/antibiotics12010075

68. Harmon B (1998) Avian heterophils in inflammation and disease resistance. Poult Sci 77:972–977. https://doi.org/10.1093/ps/77.7.972

69. Cuperus T, van Dijk A, Dwars RM, Haagsman HP (2016) Localization and developmental expression of two chicken host defense peptides: cathelicidin-2 and avian β-defensin 9. Dev Comp Immunol 61:48–59. https://doi.org/10.1016/j.dci.2016.03.008

70. van Dijk A, Tersteeg-Zijderveld MHG, Tjeerdsma-van Bokhoven JLM, Jansman AJM, Veldhuizen EJA, Haagsman HP (2009) Chicken heterophils are recruited to the site of Salmonella infection and release antibacterial mature Cathelicidin-2 upon stimulation with LPS. Mol Immunol 46:1517–1526. https://doi.org/10.1016/j.molimm.2008.12.015

71. van Dijk A, Herrebout M, Tersteeg-Zijderveld MHG, Tjeerdsma-van Bokhoven JLM, Bleumink-Pluym N, Jansman AJM, Veldhuizen EJA, Haagsman HP (2012) Campylobacter jejuni is highly susceptible to killing by chicken host defense peptide cathelicidin-2 and suppresses intestinal cathelicidin-2 expression in young broilers. Vet Microbiol 160:347–354. https://doi.org/10.1016/j.vetmic.2012.05.034

72. Dijk A van, Eldik M van, Veldhuizen EJA, Bokhoven HLMT, Zoete MR de, Bikker FJ, Haagsman HP (2016) Immunomodulatory and Anti-Inflammatory Activities of Chicken Cathelicidin-2 Derived Peptides. PLOS ONE 11:e0147919. https://doi.org/10.1371/journal.pone.0147919

73. Kraaij MD, van Dijk A, Scheenstra MR, van Harten RM, Haagsman HP, Veldhuizen EJA (2020) Chicken CATH-2 Increases Antigen Presentation Markers on Chicken Monocytes and Macrophages. Protein Pept Lett 27:60–66. https://doi.org/10.2174/0929866526666190730125525

74. Kraaij MD, van Dijk A, Haagsman HP (2017) CATH-2 and LL-37 increase mannose receptor expression, antigen presentation and the endocytic capacity of chicken mononuclear phagocytes. Mol Immunol 90:118–125. https://doi.org/10.1016/j.molimm.2017.07.005

75. Scheenstra MR, van den Belt M, Tjeerdsma-van Bokhoven JLM, Schneider VAF, Ordonez SR, van Dijk A, Veldhuizen EJA, Haagsman HP (2019) Cathelicidins PMAP-36, LL-37 and CATH-2 are similar peptides with different modes of action. Sci Rep 9:4780. https://doi.org/10.1038/s41598-019-41246-6

76. van Harten RM, Veldhuizen EJA, Haagsman HP, Scheenstra MR (2022) The cathelicidin CATH-2 efficiently neutralizes LPS- and E. coli-induced activation of porcine bone marrow derived macrophages. Vet Immunol Immunopathol 244:110369. https://doi.org/10.1016/j.vetimm.2021.110369

77. Coorens M, Schneider VAF, Groot AM de, Dijk A van, Meijerink M, Wells JM, Scheenstra MR, Veldhuizen EJA, Haagsman HP (2017) Cathelicidins Inhibit Escherichia coli–Induced TLR2 and TLR4 Activation in a Viability-Dependent Manner. J Immunol 199:1418–1428. https://doi.org/10.4049/jimmunol.1602164

78. Kannaki TR, Reddy MR, Shanmugam M, Verma PC, Sharma RP (2010) Chicken toll-like receptors and their role in immunity. Worlds Poult Sci J 66:727–738. https://doi.org/10.1017/S0043933910000693

79. van Harten RM, Tjeerdsma-van Bokhoven JLM, de Greeff A, Balhuizen MD, van Dijk A, Veldhuizen EJA, Haagsman HP, Scheenstra MR (2022) d-enantiomers of CATH-2 enhance the response of macrophages against Streptococcus suis serotype 2. J Adv Res 36:101–112. https://doi.org/10.1016/j.jare.2021.05.009

80. Schneider VAF, van Dijk A, van der Sar AM, Kraaij MD, Veldhuizen EJA, Haagsman HP (2016) Prophylactic administration of chicken cathelicidin-2 boosts zebrafish embryonic innate immunity. Dev Comp Immunol 60:108–114. https://doi.org/10.1016/j.dci.2016.02.023

81. Banaschewski BJH, Baer B, Arsenault C, Jazey T, Veldhuizen EJA, Delport J, Gooyers T, Lewis JF, Haagsman HP, Veldhuizen RAW, Yamashita C (2017) The Antibacterial and Anti-inflammatory Activity of Chicken Cathelicidin-2 combined with Exogenous Surfactant for the Treatment of Cystic Fibrosis-Associated Pathogens. Sci Rep 7:15545. https://doi.org/10.1038/s41598-017-15558-4

82. Cuperus T, van Dijk A, Matthijs MGR, Veldhuizen EJA, Haagsman HP (2016) Protective effect of in ovo treatment with the chicken cathelicidin analog D-CATH-2 against avian pathogenic E. coli. Sci Rep 6:26622. https://doi.org/10.1038/srep26622

83. Verheije MH, Coorens M, Weerts EAWS, Berends AJ, van Harten RM, Angel M, Kooij J, Ordonez SR, van Beurden SJ, van Dijk A, Haagsman HP, Veldhuizen EJA (2022) Antiviral activity of selected cathelicidins against infectious bronchitis virus. Pept Sci 114:e24234. https://doi.org/10.1002/pep2.24234

84. Niyonsaba F, Madera L, Afacan N, Okumura K, Ogawa H, Hancock REW (2013) The innate defense regulator peptides IDR-HH2, IDR-1002, and IDR-1018 modulate human neutrophil functions. J Leukoc Biol 94:159–170. https://doi.org/10.1189/jlb.1012497

85. Rivas-Santiago B, Castañeda-Delgado JE, Santiago CER, Waldbrook M, González-Curiel I, León–Contreras JC, Enciso-Moreno JA, Villar V del, Mendez-Ramos J, Hancock REW, Hernandez-Pando R (2013) Ability of Innate Defence Regulator Peptides IDR-1002, IDR-HH2 and IDR-1018 to Protect against Mycobacterium tuberculosis Infections in Animal Models. PLOS ONE 8:e59119. https://doi.org/10.1371/journal.pone.0059119

86. Nijnik A, Madera L, Ma S, Waldbrook M, Elliott MR, Easton DM, Mayer ML, Mullaly SC, Kindrachuk J, Jenssen H, Hancock REW (2010) Synthetic cationic peptide IDR-1002 provides protection against bacterial infections through chemokine induction and enhanced leukocyte recruitment. J Immunol Baltim Md 1950 184:2539–2550. https://doi.org/10.4049/jimmunol.0901813

87. Pena OM, Afacan N, Pistolic J, Chen C, Madera L, Falsafi R, Fjell CD, Hancock REW (2013) Synthetic Cationic Peptide IDR-1018 Modulates Human Macrophage Differentiation. PLOS ONE 8:e52449. https://doi.org/10.1371/journal.pone.0052449

88. Achtman AH, Pilat S, Law CW, Lynn DJ, Janot L, Mayer ML, Ma S, Kindrachuk J, Finlay BB, Brinkman FSL, Smyth GK, Hancock REW, Schofield L (2012) Effective Adjunctive Therapy by an Innate Defense Regulatory Peptide in a Preclinical Model of Severe Malaria. Sci Transl Med 4:135ra64-135ra64. https://doi.org/10.1126/scitranslmed.3003515

89. Sousa MGC, Xavier PD, Cantuária AP de C, Porcino RA, Almeida JA, Franco OL, Rezende TMB (2021) Host defense peptide IDR-1002 associated with ciprofloxacin as a new antimicrobial and immunomodulatory strategy for dental pulp revascularization therapy. Microb Pathog 152:104634. https://doi.org/10.1016/j.micpath.2020.104634

90. Kirchhelle C (2018) Pharming animals: a global history of antibiotics in food production (1935–2017). Palgrave Commun 4:1–13. https://doi.org/10.1057/s41599-018-0152-2

91. Castanon JIR (2007) History of the Use of Antibiotic as Growth Promoters in European Poultry Feeds. Poult Sci 86:2466–2471. https://doi.org/10.3382/ps.2007-00249

92. Rodrigues G, Souza Santos L, Franco OL (2022) Antimicrobial Peptides Controlling Resistant Bacteria in Animal Production. Front Microbiol 13:. https://doi.org/10.3389/fmicb.2022.874153

93. Waliaula PK, Kiarie EG, Diarra MS (2024) Predisposition factors and control strategies of avian pathogenic Escherichia coli in laying hens. Front Vet Sci 11:. https://doi.org/10.3389/fvets.2024.1474549

94. Emami NK, Dalloul RA (2021) Centennial Review: Recent developments in host-pathogen interactions during necrotic enteritis in poultry. Poult Sci 100:101330. https://doi.org/10.1016/j.psj.2021.101330

95. Xu Y, Wang Q, Dong M, Song H, Hang B, Sun Y, Zhang H, Hu J (2023) Evaluation of the efficacy of the antimicrobial peptide HJH-3 in chickens infected with Salmonella Pullorum. Front Microbiol 14:. https://doi.org/10.3389/fmicb.2023.1102789

96. Kursa O, Tomczyk G, Sieczkowska A, Kostka S, Sawicka-Durkalec A (2024) Mycoplasma gallisepticum and Mycoplasma synoviae in Turkeys in Poland. Pathogens 13:78. https://doi.org/10.3390/pathogens13010078

97. Nhung NT, Chansiripornchai N, Carrique-Mas JJ (2017) Antimicrobial Resistance in Bacterial Poultry Pathogens: A Review. Front Vet Sci 4:. https://doi.org/10.3389/fvets.2017.00126

98. Singer RS, Hofacre CL (2006) Potential Impacts of Antibiotic Use in Poultry Production. Avian Dis 50:161–172. https://doi.org/10.1637/7569-033106R.1

99. Bhattarai RK, Basnet HB, Dhakal IP, Devkota B (2024) Antimicrobial resistance of avian pathogenic Escherichia coli isolated from broiler, layer, and breeder chickens. Vet World 17:480–499. https://doi.org/10.14202/vetworld.2024.480-499

100. Ali HR, Hefny EG, Koraney NF, Ali SF, AbdAllah MI, Fadel MA, Elnomrosy SM, Shahein MA (2025) Antibiotic residues correlate with antibiotic resistance of Salmonella typhimurium isolated from edible chicken meat. Sci Rep 15:15165. https://doi.org/10.1038/s41598-025-98189-4

101. Andrew Selaledi L, Mohammed Hassan Z, Manyelo TG, Mabelebele M (2020) The Current Status of the Alternative Use to Antibiotics in Poultry Production: An African Perspective. Antibiotics 9:594. https://doi.org/10.3390/antibiotics9090594

102. Muneeb M, Khan EU, Ali M, Haque MNU, Khan MUZ, Ahmad S (2025) Comparative Effects of Antibiotic and Antimicrobial Peptide on Growth Performance, Gut Morphology, Intestinal Lesion Score, Ileal Microbial Counts, and Immune Status in Broilers Challenged with Necrotic Enteritis. Probiotics Antimicrob Proteins. https://doi.org/10.1007/s12602-025-10448-y

103. Kogut MH, Lee A, Santin E (2020) Microbiome and pathogen interaction with the immune system. Poult Sci 99:1906–1913. https://doi.org/10.1016/j.psj.2019.12.011

104. Jha R, Fouhse JM, Tiwari UP, Li L, Willing BP (2019) Dietary Fiber and Intestinal Health of Monogastric Animals. Front Vet Sci 6:

105. Maswanganye GMT, Liu B, Che D, Han R (2021) Review: Effects of Dietary Fiber Levels and Composition on the Intestinal Health of Finishing Pigs. Open J Anim Sci 11:384–398. https://doi.org/10.4236/ojas.2021.113028

106. Wickramasuriya SS, Park I, Lee K, Lee Y, Kim WH, Nam H, Lillehoj HS (2022) Role of Physiology, Immunity, Microbiota, and Infectious Diseases in the Gut Health of Poultry. Vaccines 10:172. https://doi.org/10.3390/vaccines10020172

107. Scanes CG, Pierzchala-Koziec K (2014) Biology of the Gastrointestinal Tract in Poultry. Avian Biol Res 7:193–222. https://doi.org/10.3184/175815514X14162292284822

108. van Dijk A, Veldhuizen EJA, Kalkhove SIC, Tjeerdsma-van Bokhoven JLM, Romijn RA, Haagsman HP (2007) The β-Defensin Gallinacin-6 Is Expressed in the Chicken Digestive Tract and Has Antimicrobial Activity against Food-Borne Pathogens. Antimicrob Agents Chemother 51:912–922. https://doi.org/10.1128/aac.00568-06

109. Tabata H, Yasugi S (1998) Tissue interaction regulates expression of a spasmolytic polypeptide gene in chicken stomach epithelium. Dev Growth Differ 40:519–526. https://doi.org/10.1046/j.1440-169X.1998.t01-3-00006.x

110. Turk DE (1982) The Anatomy of the Avian Digestive Tract as Related to Feed Utilization1. Poult Sci 61:1225–1244. https://doi.org/10.3382/ps.0611225

111. Smirnov A, Perez R, Amit-Romach E, Sklan D, Uni Z (2005) Mucin Dynamics and Microbial Populations in Chicken Small Intestine Are Changed by Dietary Probiotic and Antibiotic Growth Promoter Supplementation. J Nutr 135:187–192. https://doi.org/10.1093/jn/135.2.187

112. Hong YH, Song W, Lee SH, Lillehoj HS (2012) Differential gene expression profiles of β-defensins in the crop, intestine, and spleen using a necrotic enteritis model in 2 commercial broiler chicken lines. Poult Sci 91:1081–1088. https://doi.org/10.3382/ps.2011-01948

113. Abreu MT (2003) Immunologic regulation of toll-like receptors in gut epithelium. Curr Opin Gastroenterol 19:559

114. Vaughn LE, Holt PS, Moore RW, Gast RK (2006) Enhanced gross visualization of chicken Peyer's patch: novel staining technique applied to fresh tissue specimens. Avian Dis 50:298–302. https://doi.org/10.1637/7467-110305R.1

115. Lillehoj HS, Trout JM (1996) Avian gut-associated lymphoid tissues and intestinal immune responses to Eimeria parasites. Clin Microbiol Rev 9:349–360. https://doi.org/10.1128/cmr.9.3.349

116. Maldonado-Contreras AL, McCormick BA (2011) Intestinal epithelial cells and their role in innate mucosal immunity. Cell Tissue Res 343:5–12. https://doi.org/10.1007/s00441-010-1082-5

117. Dahan S, Roth-Walter F, Arnaboldi P, Agarwal S, Mayer L (2007) Epithelia: lymphocyte interactions in the gut. Immunol Rev 215:243–253. https://doi.org/10.1111/j.1600-065X.2006.00484.x

118. Chelakkot C, Ghim J, Ryu SH (2018) Mechanisms regulating intestinal barrier integrity and its pathological implications. Exp Mol Med 50:1–9. https://doi.org/10.1038/s12276-018-0126-x

119. Robinson MW, Harmon C, O'Farrelly C (2016) Liver immunology and its role in inflammation and homeostasis. Cell Mol Immunol 13:267–276. https://doi.org/10.1038/cmi.2016.3

120. Rui L (2014) Energy Metabolism in the Liver. Compr Physiol 4:177–197. https://doi.org/10.1002/cphy.c130024

121. Crosby WH (1955) The metabolism of hemoglobin and bile pigment in hemolytic disease. Am J Med 18:112–122. https://doi.org/10.1016/0002-9343(55)90208-4

122. Stoll B, Gerok W, Lang F, Häussinger D (1992) Liver cell volume and protein synthesis. Biochem J 287:217–222. https://doi.org/10.1042/bj2870217

123. Denbow DM (2015) Gastrointestinal Anatomy and Physiology. In: Scanes CG (ed) Sturkie's Avian Physiology (Sixth Edition), 5th ed. Academic Press, San Diego, pp 299–304

124. Wisse E, Braet F, Luo D, De Zanger R, Jans D, Crabbe E, Vermoesen A (1996) Structure and Function of Sinusoidal Lining Cells in the Liver. Toxicol Pathol 24:100–111. https://doi.org/10.1177/019262339602400114

125. Takeuchi O, Akira S (2010) Pattern Recognition Receptors and Inflammation. Cell 140:805–820. https://doi.org/10.1016/j.cell.2010.01.022

126. Kubes P, Mehal WZ (2012) Sterile Inflammation in the Liver. Gastroenterology 143:1158–1172. https://doi.org/10.1053/j.gastro.2012.09.008

127. Bilzer M, Roggel F, Gerbes AL (2006) Role of Kupffer cells in host defense and liver disease. Liver Int 26:1175–1186. https://doi.org/10.1111/j.1478-3231.2006.01342.x

128. Townes CL, Michailidis G, Nile CJ, Hall J (2004) Induction of cationic chicken liver-expressed antimicrobial peptide 2 in response to Salmonella enterica infection. Infect Immun 72:6987–6993. https://doi.org/10.1128/IAI.72.12.6987-6993.2004

129. Zhao C, Nguyen T, Liu L, Sacco RE, Brogden KA, Lehrer RI (2001) Gallinacin-3, an inducible epithelial beta-defensin in the chicken. Infect Immun 69:2684–2691. https://doi.org/10.1128/IAI.69.4.2684-2691.2001

130. van Dijk A, Veldhuizen EJA, van Asten AJAM, Haagsman HP (2005) CMAP27, a novel chicken cathelicidin-like antimicrobial protein. Vet Immunol Immunopathol 106:321–327. https://doi.org/10.1016/j.vetimm.2005.03.003

131. Lauridsen C (2019) From oxidative stress to inflammation: redox balance and immune system. Poult Sci 98:4240–4246. https://doi.org/10.3382/ps/pey407

132. Harrison RG, Greenman MJ, Mall FP, Jackson CM (1907) Observations of the living developing nerve fiber. Anat Rec 1:116–128. https://doi.org/10.1002/ar.1090010503

133. Carrel A, Burrows MT (1911) An addition to the technique of the cultivation of tissues in vitro. J Exp Med 14:244–247

134. Carrel A, Burrows MT (1911) Cultivation of tissues in vitro and its technique. J Exp Med 13:387–396

135. Rodríguez-Hernández CO, Torres-García SE, Olvera-Sandoval C, Ramírez-Castillo FY, Muro AL, Avelar-Gonzalez FJ, Guerrero-Barrera AL (2014) Cell Culture: History, Development and Prospects. Int J Curr Res Acad Rev 2347–3215:188–200

136. White PR (1946) Cultivation of animal tissues in vitro in nutrients of precisely known constitution. Growth 10:231–289

137. Earle WR, Schilling EL, Stark TH, Straus NP, Brown MF, Shelton E (1943) Production of Malignancy in Vitro. IV. The Mouse Fibroblast Cultures and Changes Seen in the Living Cells. JNCI J Natl Cancer Inst 4:165–212. https://doi.org/10.1093/jnci/4.2.165

138. Sanford KK, Earle WR, Likely GD (1948) The growth in vitro of single isolated tissue cells. J Natl Cancer Inst 9:229–246

139. Kaur G, Dufour JM (2012) Cell lines: Valuable tools or useless artifacts. Spermatogenesis 2:1–5. https://doi.org/10.4161/spmg.19885

140. Nelson-Rees WA, Daniels DW, Flandermeyer RR (1981) Cross-Contamination of Cells in Culture. Science 212:446–452. https://doi.org/10.1126/science.6451928

141. Stacey G (2006) Primary Cell Cultures and Immortal Cell Lines. In: Encyclopedia of Life Sciences. John Wiley & Sons, Ltd

142. Duval K, Grover H, Han L-H, Mou Y, Pegoraro AF, Fredberg J, Chen Z (2017) Modeling Physiological Events in 2D vs. 3D Cell Culture. Physiol Bethesda Md 32:266–277. https://doi.org/10.1152/physiol.00036.2016

143. Lawrence TS, Beers WH, Gilula NB (1978) Transmission of hormonal stimulation by cell-to-cell communication. Nature 272:501–506. https://doi.org/10.1038/272501a0

144. Paschos NK, Brown WE, Eswaramoorthy R, Hu JC, Athanasiou KA (2015) Advances in tissue engineering through stem cell-based co-culture. J Tissue Eng Regen Med 9:488–503. https://doi.org/10.1002/term.1870

145. Matis G, Sebők C, Horváth D, Márton R, Mackei M, Vörösházi J, Kemeny A, Zsuzsanna N, Varga I, Tráj P (2024) Miniature chicken ileal explant culture to investigate the inflammatory response induced by pathogen associated molecular patterns. SSRN Electron J. https://doi.org/10.2139/ssrn.4685306

146. Hooper LV, Littman DR, Macpherson AJ (2012) Interactions between the microbiota and the immune system. Science 336:1268–1273. https://doi.org/10.1126/science.1223490

147. Browning TH, Trier JS (1969) Organ culture of mucosal biopsies of human small intestine. J Clin Invest 48:1423–1432. https://doi.org/10.1172/JCI106108

148. Costa MO, Harding JCS, Hill JE (2016) Development and evaluation of a porcine in vitro colon organ culture technique. In Vitro Cell Dev Biol Anim 52:942–952. https://doi.org/10.1007/s11626-016-0060-y

149. Randall KJ, Turton J, Foster JR (2011) Explant culture of gastrointestinal tissue: a review of methods and applications. Cell Biol Toxicol 27:267–284. https://doi.org/10.1007/s10565-011-9187-5

150. Zhang Q, Eicher SD, Ajuwon KM, Applegate TJ (2017) Development of a chicken ileal explant culture model for measurement of gut inflammation induced by lipopolysaccharide. Poult Sci 96:3096–3103. https://doi.org/10.3382/ps/pex160

151. Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT (2012) PAMPs and DAMPs: signal 0s that spur autophagy and immunity. Immunol Rev 249:158–175. https://doi.org/10.1111/j.1600-065X.2012.01146.x

152. Dickson K, Lehmann C (2019) Inflammatory Response to Different Toxins in Experimental Sepsis Models. Int J Mol Sci 20:. https://doi.org/10.3390/ijms20184341

153. Ginsburg I (2002) Role of lipoteichoic acid in infection and inflammation. Lancet Infect Dis 2:171–179. https://doi.org/10.1016/S1473-3099(02)00226-8

154. Kiziltas S (2016) Toll-like receptors in pathophysiology of liver diseases. World J Hepatol 8:1354–1369. https://doi.org/10.4254/wjh.v8.i32.1354

155. Kalaiyarasu S, Bhatia S, Mishra N, Sood R, Kumar M, SenthilKumar D, Bhat S, Dass Prakash M (2016) Elevated level of pro inflammatory cytokine and chemokine expression in chicken bone marrow and monocyte derived dendritic cells following LPS induced maturation. Cytokine 85:140–147. https://doi.org/10.1016/j.cyto.2016.06.022

156. Zhang Q, Eicher SD, Ajuwon KM, Applegate TJ (2017) Development of a chicken ileal explant culture model for measurement of gut inflammation induced by lipopolysaccharide. Poult Sci 96:3096–3103. https://doi.org/10.3382/ps/pex160

157. Farnell MB, He H, Kogut MH (2003) Differential activation of signal transduction pathways mediating oxidative burst by chicken heterophils in response to stimulation with lipopolysaccharide and lipoteichoic acid. Inflammation 27:225–231. https://doi.org/10.1023/a:1025088514676

158. Kim J, Kim S, Jeon S, Hui Z, Kim Y, Im Y, Lim W, Kim C, Choi H, Kim O (2015) Anti-inflammatory effects of zinc in PMA-treated human gingival fibroblast cells. Med Oral Patol Oral Cir Bucal 20:e180–e187. https://doi.org/10.4317/medoral.19896

159. Zhang G, Liu X, Wang C, Qu L, Deng J, Wang H, Qin Z (2013) Resolution of PMA-Induced Skin Inflammation Involves Interaction of IFN-γ and ALOX15. Mediators Inflamm 2013:. https://doi.org/10.1155/2013/930124

160. He H, Farnell MB, Kogut MH (2003) Inflammatory agonist stimulation and signal pathway of oxidative burst in neonatal chicken heterophils. Comp Biochem Physiol A Mol Integr Physiol 135:177–184. https://doi.org/10.1016/s1095-6433(03)00049-7

161. Mackei M, Molnár A, Nagy S, Pál L, Kővágó C, Gálfi P, Dublecz K, Husvéth F, Neogrády Z, Mátis G (2020) Effects of Acute Heat Stress on a Newly Established Chicken Hepatocyte—Nonparenchymal Cell Co-Culture Model. Animals 10:409. https://doi.org/10.3390/ani10030409

162. Mátis G, Sebők C, Horváth DG, Márton RA, Mackei M, Vörösházi J, Kemény Á, Neogrády Z, Varga I, Tráj P (2024) Miniature chicken ileal explant culture to investigate the inflammatory response induced by pathogen associated molecular patterns

163. Kogut MH (2019) The effect of microbiome modulation on the intestinal health of poultry. Anim Feed Sci Technol 250:32–40. https://doi.org/10.1016/j.anifeedsci.2018.10.008

164. Mackei M, Orbán K, Molnár A, Pál L, Dublecz K, Husvéth F, Neogrády Z, Mátis G (2020) Cellular effects of T-2 toxin on primary hepatic cell culture models of chickens. Toxins 12:1–14. https://doi.org/10.3390/toxins12010046

165. Mátis G, Kulcsár A, Petrilla J, Talapka P, Neogrády Z (2017) Porcine hepatocyte–Kupffer cell co-culture as an in vitro model for testing the efficacy of anti-inflammatory substances. J Anim Physiol Anim Nutr 101:201–207. https://doi.org/10.1111/jpn.12547

166. Kim J-K, Lee S-M, Suk K, Lee W-H (2011) A Novel Pathway Responsible for Lipopolysaccharide-Induced Translational Regulation of TNF-α and IL-6 Expression Involves Protein Kinase C and Fascin. J Immunol 187:6327–6334. https://doi.org/10.4049/jimmunol.1100612

167. Gao B, Jeong W-I, Tian Z (2008) Liver: An organ with predominant innate immunity. Hepatol Baltim Md 47:729–736. https://doi.org/10.1002/hep.22034

168. Keestra AM, Putten JPM van (2008) Unique Properties of the Chicken TLR4/MD-2 Complex: Selective Lipopolysaccharide Activation of the MyD88-Dependent Pathway. J Immunol 181:4354–4362. https://doi.org/10.4049/jimmunol.181.6.4354

169. Seki E, Brenner DA (2008) Toll-like receptors and adaptor molecules in liver disease: Update. Hepatology 48:322–335. https://doi.org/10.1002/hep.22306

170. Esnault E, Bonsergent C, Larcher T, Bed'hom B, Vautherot J-F, Delaleu B, Guigand L, Soubieux D, Marc D, Quéré P (2011) A novel chicken lung epithelial cell line: Characterization and response to low pathogenicity avian influenza virus. Virus Res 159:32–42. https://doi.org/10.1016/j.virusres.2011.04.022

171. Lian L, Ciraci C, Chang G, Hu J, Lamont SJ (2012) NLRC5 knockdown in chicken macrophages alters response to LPS and poly (I:C) stimulation. BMC Vet Res 8:23. https://doi.org/10.1186/1746-6148-8-23

172. Abdel-Mageed AM, Isobe N, Yoshimura Y (2014) Effects of different TLR ligands on the expression of proinflammatory cytokines and avian β-defensins in the uterine and vaginal tissues of laying hens. Vet Immunol Immunopathol 162:132–141. https://doi.org/10.1016/j.vetimm.2014.10.013

173. Kamimura T, Isobe N, Yoshimura Y (2017) Effects of inhibitors of transcription factors, nuclear factor-κB and activator protein 1, on the expression of proinflammatory cytokines and chemokines induced by stimulation with Toll-like receptor ligands in hen vaginal cells. Poult Sci 96:723–730. https://doi.org/10.3382/ps/pew366

174. Rajput IR, Hussain A, Li YL, Zhang X, Xu X, Long MY, You DY, Li WF (2014) Saccharomyces boulardii and Bacillus subtilis B10 modulate TLRs mediated signaling to induce immunity by chicken BMDCs. J Cell Biochem 115:189–198. https://doi.org/10.1002/jcb.24650

175. Cheng P, Wang T, Li W, Muhammad I, Wang H, Sun X, Yang Y, Li J, Xiao T, Zhang X (2017) Baicalin Alleviates Lipopolysaccharide-Induced Liver Inflammation in Chicken by Suppressing TLR4-Mediated NF-κB Pathway. Front Pharmacol 8:547. https://doi.org/10.3389/fphar.2017.00547

176. Seno K, Munakata Y, Sano M, Kawahara-Miki R, Takahashi H, Ohkuchi A, Iwata H, Kuwayama T, Shirasuna K (2018) Aggregation of Human Trophoblast Cells into Three-Dimensional Culture System Enhances Anti-Inflammatory Characteristics through Cytoskeleton Regulation. Int J Mol Sci 19:2322. https://doi.org/10.3390/ijms19082322

177. Rola-Pleszczynski M, Stankova J (1992) Leukotriene B4 Enhances Interleukin-6 (IL-6) Production and IL-6 Messenger RNA Accumulation in Human Monocytes In Vitro: Transcriptional and Posttranscriptional Mechanisms. Blood 80:1004–1011. https://doi.org/10.1182/blood.V80.4.1004.1004

178. Niyonsaba F, Someya A, Hirata M, Ogawa H, Nagaoka I (2001) Evaluation of the effects of peptide antibiotics human β-defensins-1/-2 and LL-37 on histamine release and prostaglandin D2 production from mast cells. Eur J Immunol 31:1066–1075. https://doi.org/10.1002/1521-4141(200104)31:4<1066::AID-IMMU1066>3.0.CO;2-#

179. Lau YE, Bowdish DME, Cosseau C, Hancock REW, Davidson DJ (2006) Apoptosis of airway epithelial cells: human serum sensitive induction by the cathelicidin LL-37. Am J Respir Cell Mol Biol 34:399–409. https://doi.org/10.1165/rcmb.2005-0170OC

180. Chen X, Niyonsaba F, Ushio H, Hara M, Yokoi H, Matsumoto K, Saito H, Nagaoka I, Ikeda S, Okumura K, Ogawa H (2007) Antimicrobial peptides human β-defensin (hBD)-3 and hBD-4 activate mast cells and increase skin vascular permeability. Eur J Immunol 37:434–444. https://doi.org/10.1002/eji.200636379

181. Fjell CD, Hiss JA, Hancock REW, Schneider G (2012) Designing antimicrobial peptides: form follows function. Nat Rev Drug Discov 11:37–51. https://doi.org/10.1038/nrd3591

182. Johansson J, Gudmundsson GH, Rottenberg ME, Berndt KD, Agerberth B (1998) Conformation-dependent Antibacterial Activity of the Naturally Occurring Human Peptide LL-37 *. J Biol Chem 273:3718–3724. https://doi.org/10.1074/jbc.273.6.3718

183. Oren Z, Lerman JC, Gudmundsson GH, Agerberth B, Shai Y (1999) Structure and organization of the human antimicrobial peptide LL-37 in phospholipid membranes: relevance to the molecular basis for its non-cell-selective activity. Biochem J 341:501–513

184. Sebők C, Walmsley S, Tráj P, Mackei M, Vörösházi J, Petrilla J, Kovács L, Kemény Á, Neogrády Z, Mátis G (2022) Immunomodulatory effects of chicken cathelicidin-2 on a primary hepatic cell co-culture model. PLOS ONE 17:e0275847. https://doi.org/10.1371/journal.pone.0275847

185. Peng L, Scheenstra MR, van Harten RM, Haagsman HP, Veldhuizen EJA (2020) The immunomodulatory effect of cathelicidin-B1 on chicken macrophages. Vet Res 51:122. https://doi.org/10.1186/s13567-020-00849-y

186. van Dijk A, van Eldik M, Veldhuizen EJA, Tjeerdsma-van Bokhoven HLM, de Zoete MR, Bikker FJ, Haagsman HP (2016) Immunomodulatory and Anti-Inflammatory Activities of Chicken Cathelicidin-2 Derived Peptides. PLOS ONE 11:e014791919. https://doi.org/10.1371/JOURNAL.PONE.0147919

187. Schneider VAF, Haagsman HP (2016) Shedding light on antibacterial activities of cathelicidins. Utrecht University

188. Poh TY, Pease J, Young JR, Bumstead N, Kaiser P (2008) Re-evaluation of Chicken CXCR1 Determines the True Gene Structure: CXCLi1 (K60) AND CXCLi2 (CAF/INTERLEUKIN-8) ARE LIGANDS FOR THIS RECEPTOR*. J Biol Chem 283:16408–16415. https://doi.org/10.1074/jbc.M800998200

189. Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N, Matsushima K (1994) Essential involvement of interleukin-8 (IL-8) in acute inflammation. J Leukoc Biol 56:559–564

190. Hutchins AP, Diez D, Miranda-Saavedra D (2013) The IL-10/STAT3-mediated anti-inflammatory response: recent developments and future challenges. Brief Funct Genomics 12:489–498. https://doi.org/10.1093/bfgp/elt028

191. Lin F-C, Young HA (2013) The talented interferon-gamma. 2013:. https://doi.org/10.4236/abb.2013.47A3002

192. Pixley FJ, Stanley ER (2004) CSF-1 regulation of the wandering macrophage: complexity in action. Trends Cell Biol 14:628–638. https://doi.org/10.1016/j.tcb.2004.09.016

193. Lin F-C, Young HA (2013) The talented interferon-gamma. Adv Biosci Biotechnol 4:6–13. https://doi.org/10.4236/ABB.2013.47A3002

194. Acosta J, Roa F, González-Chavarría I, Astuya A, Maura R, Montesino R, Muñoz C, Camacho F, Saavedra P, Valenzuela A, Sánchez O, Toledo JR (2019) In vitro immunomodulatory activities of peptides derived from Salmo salar NK-lysin and cathelicidin in fish cells. Fish Shellfish Immunol 88:587–594. https://doi.org/10.1016/J.FSI.2019.03.034

195. Harada A, Mukaida N, Sekido N, Akahoshi T, Wada T, Matsushima K (1994) Essential involvement of interleukin-8 (IL-8) in acute inflammation. Artic J Leukoc Biol 56:559–564. https://doi.org/10.1002/jlb.56.5.559

196. Laforge M, Elbim C, Frère C, Hémadi M, Massaad C, Nuss P, Benoliel J-J, Becker C (2020) Tissue damage from neutrophil-induced oxidative stress in COVID-19. Nat Rev Immunol 2020 209 20:515–516. https://doi.org/10.1038/s41577-020-0407-1

197. Brat DJ, Bellail AC, Meir EG Van (2005) The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. Neuro-Oncol 7:133. https://doi.org/10.1215/S1152851704001061

198. Mookherjee N, Brown KL, Bowdish DME, Doria S, Falsafi R, Hokamp K, Roche FM, Mu R, Doho GH, Pistolic J, Powers J-P, Bryan J, Brinkman FSL, Hancock REW (2006) Modulation of the TLR-Mediated Inflammatory Response by the Endogenous Human Host Defense Peptide LL-37. J Immunol 176:2455–2464. https://doi.org/10.4049/jimmunol.176.4.2455

199. Oudhoff MJ, Blaauboer ME, Nazmi K, Scheres N, Bolscher JGM, Veerman ECI (2010) The role of salivary histatin and the human cathelicidin LL-37 in wound healing and innate immunity. Biol Chem 391:541–548. https://doi.org/10.1515/BC.2010.057

200. Hutchins AP, Diez D, Miranda-Saavedra D (2013) The IL-10/STAT3-mediated anti-inflammatory response: recent developments and future challenges. Brief Funct Genomics 12:489–498. https://doi.org/10.1093/BFGP/ELT028

201. Fixe P, Praloran V (1998) M-CSF: Haematopoietic growth factor or inflammatory cytokine? Cytokine 10:32–37

202. Pixley FJ, Stanley ER (2004) CSF-1 regulation of the wandering macrophage: complexity in action. Trends Cell Biol 14:628–638. https://doi.org/10.1016/J.TCB.2004.09.016

203. Yeung Y-G, Stanley ER (2003) Proteomic Approaches to the Analysis of Early Events in Colony-stimulating Factor-1 Signal Transduction *. Mol Cell Proteomics 2:1143–1155. https://doi.org/10.1074/mcp.R300009-MCP200

204. Xu W, Schlagwein N, Roos A, van denBerg TK, Daha MR, van Kooten C (2007) Human peritoneal macrophages show functional characteristics of M-CSF-driven anti-inflammatory type 2 macrophages. Eur J Immunol 37:1594–1599. https://doi.org/10.1002/EJI.200737042

205. Norman JG, Fink GW, Denham W, Yang J, Carter G, Sexton C, Falkner J, Gower WR, Franz MG (1997) Tissue-specific cytokine production during experimental acute pancreatitis: A probable mechanism for distant organ dysfunction. Dig Dis Sci 42:1783–1788

206. Hoek JB, Pastorino JG (2002) Ethanol, oxidative stress, and cytokine-induced liver cell injury. Alcohol 27:63–68. https://doi.org/10.1016/S0741-8329(02)00215-X

207. Butterfield TA, Best TM, Merrick MA (2006) The Dual Roles of Neutrophils and Macrophages in Inflammation: A Critical Balance Between Tissue Damage and Repair. J Athl Train 41:465

208. Cao X, Wang Y, Wu C, Li X, Fu Z, Yang M, Bian W, Wang S, Song Y, Tang J, Yang X (2018) Cathelicidin-OA1, a novel antioxidant peptide identified from an amphibian, accelerates skin wound healing. Sci Rep 8:1–15. https://doi.org/10.1038/s41598-018-19486-9

209. Harten RM Van, Woudenbergh E Van, Dijk A Van, Haagsman HP (2018) Cathelicidins: Immunomodulatory Antimicrobials. Vaccines 6:63. https://doi.org/10.3390/VACCINES6030063

210. Peng M, Liu Y, Xu Y, Li L, Li Y, Yang H (2021) Cathelicidin-WA ameliorates diabetic cardiomyopathy by inhibiting the NLRP3 inflammasome. Cell Cycle 20:2278–2290. https://doi.org/10.1080/15384101.2021.1981631

211. Wong CCM, Zhang L, Li ZJ, Wu WKK, Ren SX, Chen YC, Ng TB, Cho CH (2012) Protective effects of cathelicidin-encoding Lactococcus lactis in murine ulcerative colitis. J Gastroenterol Hepatol 27:1205–1212. https://doi.org/10.1111/j.1440-1746.2012.07158.x

212. Easton DM, Nijnik A, Mayer ML, Hancock REW (2009) Potential of immunomodulatory host defense peptides as novel anti-infectives. Trends Biotechnol 27:582–590. https://doi.org/10.1016/j.tibtech.2009.07.004

213. Yanashima K, Chieosilapatham P, Yoshimoto E, Okumura K, Ogawa H, Niyonsaba F (2017) Innate defense regulator IDR-1018 activates human mast cells through G protein-, phospholipase C-, MAPK- and NF-κB-sensitive pathways. Immunol Res 65:920–931. https://doi.org/10.1007/s12026-017-8932-0

214. Wieczorek M, Jenssen H, Kindrachuk J, Scott WRP, Elliott M, Hilpert K, Cheng JTJ, Hancock REW, Straus SK (2010) Structural Studies of a Peptide with Immune Modulating and Direct Antimicrobial Activity. Chem Biol 17:970–980. https://doi.org/10.1016/j.chembiol.2010.07.007

215. Chen L, Zhang Q, Yu C, Wang F, Kong X (2020) Functional roles of CCL5/RANTES in liver disease. Liver Res 4:28–34. https://doi.org/10.1016/j.livres.2020.01.002

216. Madera L, Hancock REW (2012) Synthetic Immunomodulatory Peptide IDR-1002 Enhances Monocyte Migration and Adhesion on Fibronectin. J Innate Immun 4:553–568. https://doi.org/10.1159/000338648

217. Naito M, Hasegawa G, Takahashi K (1997) Development, differentiation, and maturation of Kupffer cells. Microsc Res Tech 39:350–364. https://doi.org/10.1002/(SICI)1097-0029(19971115)39:4<350::AID-JEMT5>3.0.CO;2-L

218. Amemiya H, Kono H, Fujii H (2011) Liver Regeneration is Impaired in Macrophage Colony Stimulating Factor Deficient Mice After Partial Hepatectomy: The Role of M-CSF-Induced Macrophages. J Surg Res 165:59–67. https://doi.org/10.1016/j.jss.2009.08.008

219. van der Does AM, Beekhuizen H, Ravensbergen B, Vos T, Ottenhoff THM, van Dissel JT, Drijfhout JW, Hiemstra PS, Nibbering PH (2010) LL-37 Directs Macrophage Differentiation toward Macrophages with a Proinflammatory Signature. J Immunol 185:1442–1449. https://doi.org/10.4049/jimmunol.1000376

220. Rőszer T (2015) Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. Mediators Inflamm 2015:e816460. https://doi.org/10.1155/2015/816460

221. Wuerth K, Lee AHY, Falsafi R, Gill EE, Hancock REW (2018) Characterization of Host Responses during Pseudomonas aeruginosa Acute Infection in the Lungs and Blood and after Treatment with the Synthetic Immunomodulatory Peptide IDR-1002. Infect Immun 87:e00661-18. https://doi.org/10.1128/IAI.00661-18

222. Wuerth KC, Falsafi R, Hancock REW (2017) Synthetic host defense peptide IDR-1002 reduces inflammation in Pseudomonas aeruginosa lung infection. PLOS ONE 12:e0187565. https://doi.org/10.1371/journal.pone.0187565

223. Huante-Mendoza A, Silva-García O, Oviedo-Boyso J, Hancock REW, Baizabal-Aguirre VM (2016) Peptide IDR-1002 Inhibits NF-κB Nuclear Translocation by Inhibition of IκBα Degradation and Activates p38/ERK1/2–MSK1-Dependent CREB Phosphorylation in Macrophages Stimulated with Lipopolysaccharide. Front Immunol 7:

224. Wu C, Xue Y, Wang P, Lin L, Liu Q, Li N, Xu J, Cao X (2014) IFN-γ primes macrophage activation by increasing phosphatase and tensin homolog via downregulation of miR-3473b. J Immunol Baltim Md 1950 193:3036–3044. https://doi.org/10.4049/jimmunol.1302379

225. Sun R, Gao B (2004) Negative regulation of liver regeneration by innate immunity (natural killer cells/interferon-γ). Gastroenterology 127:1525–1539. https://doi.org/10.1053/j.gastro.2004.08.055

226. Knight B, Lim R, Yeoh GC, Olynyk JK (2007) Interferon-gamma exacerbates liver damage, the hepatic progenitor cell response and fibrosis in a mouse model of chronic liver injury. J Hepatol 47:826–833. https://doi.org/10.1016/j.jhep.2007.06.022

227. Turner-Brannen E, Choi K-Y, Lippert DN, Cortens JP, Hancock RE, El-Gabalawy H, Mookherjee N (2011) Modulation of interleukin-1β-induced inflammatory responses by a synthetic cationic innate defence regulator peptide, IDR-1002, in synovial fibroblasts. Arthritis Res Ther 13:R129. https://doi.org/10.1186/ar3440

228. Steinmetz C, Kashyap A, Zhivkova N, Alizor H, Ernst I, Gottfried-Brand D, Janssen H, Teufel A, Schulze-Bergkamen H, Lotz J, Kuball J, Theobald M, Heise M, Lang H, Galle PR, Strand D, Strand S (2017) Activation of silent mating type information regulation 2 homolog 1 by human chorionic gonadotropin exerts a therapeutic effect on hepatic injury and inflammation. Hepatology 65:2074–2089. https://doi.org/10.1002/hep.29072

229. Shoji J, Aso H, Inada N (2017) Clinical Usefulness of Simultaneous Measurement of the Tear Levels of CCL17, CCL24, and IL-16 for the Biomarkers of Allergic Conjunctival Disorders. Curr Eye Res 42:677–684. https://doi.org/10.1080/02713683.2016.1242755

230. Silva MT (2010) When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. J Leukoc Biol 87:93–106. https://doi.org/10.1189/jlb.0809549

231. Li S, Hong M, Tan H-Y, Wang N, Feng Y (2016) Insights into the Role and Interdependence of Oxidative Stress and Inflammation in Liver Diseases. Oxid Med Cell Longev 2016:e4234061. https://doi.org/10.1155/2016/4234061

232. Schieber M, Chandel NS (2014) ROS Function in Redox Signaling and Oxidative Stress. Curr Biol 24:R453–R462. https://doi.org/10.1016/j.cub.2014.03.034

233. Çimen B, Türközkan N, Seven I, Ünlü A, Karasu Ç (2004) Impaired Na+,K+-ATPase activity as a mechanism of reactive nitrogen species-induced cytotoxicity in guinea pig liver exposed to lipopolysaccharides. Mol Cell Biochem 259:53–57. https://doi.org/10.1023/B:MCBI.0000021344.64317.a2

234. Huang Y, Li W, Su Z, Kong A-NT (2015) The complexity of the Nrf2 pathway: beyond the antioxidant response. J Nutr Biochem 26:1401–1413. https://doi.org/10.1016/j.jnutbio.2015.08.001

235. Lee I-T, Wang S-W, Lee C-W, Chang C-C, Lin C-C, Luo S-F, Yang C-M (2008) Lipoteichoic Acid Induces HO-1 Expression via the TLR2/MyD88/c-Src/NADPH Oxidase Pathway and Nrf2 in Human Tracheal Smooth Muscle Cells1. J Immunol 181:5098–5110. https://doi.org/10.4049/jimmunol.181.7.5098

236. Rushworth SA, Chen X-L, Mackman N, Ogborne RM, O'Connell MA (2005) Lipopolysaccharide-Induced Heme Oxygenase-1 Expression in Human Monocytic Cells Is Mediated via Nrf2 and Protein Kinase C. J Immunol 175:4408–4415. https://doi.org/10.4049/jimmunol.175.7.4408

237. Pletzer D, Mansour SC, Hancock REW (2018) Synergy between conventional antibiotics and anti-biofilm peptides in a murine, sub-cutaneous abscess model caused by recalcitrant ESKAPE pathogens. PLOS Pathog 14:e1007084. https://doi.org/10.1371/journal.ppat.1007084

238. de Breij A, Riool M, Cordfunke RA, Malanovic N, de Boer L, Koning RI, Ravensbergen E, Franken M, van der Heijde T, Boekema BK, Kwakman PHS, Kamp N, El Ghalbzouri A, Lohner K, Zaat SAJ, Drijfhout JW, Nibbering PH (2018) The antimicrobial peptide SAAP-148 combats drug-resistant bacteria and biofilms. Sci Transl Med 10:eaan4044. https://doi.org/10.1126/scitranslmed.aan4044

239. Haney EF, Mansour SC, Hilchie AL, de la Fuente-Núñez C, Hancock REW (2015) High throughput screening methods for assessing antibiofilm and immunomodulatory activities of synthetic peptides. Peptides 71:276–285. https://doi.org/10.1016/j.peptides.2015.03.015

240. Flemming H-C, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S (2016) Biofilms: an emergent form of bacterial life. Nat Rev Microbiol 14:563–575. https://doi.org/10.1038/nrmicro.2016.94

241. Lima LS, Ramalho SR, Sandim GC, Parisotto EB, Orlandi Sardi J de C, Rodrigues Macedo ML (2022) Prevention of hospital pathogen biofilm formation by antimicrobial peptide KWI18. Microb Pathog 172:105791. https://doi.org/10.1016/j.micpath.2022.105791

242. Ting KKY, Jongstra-Bilen J, Cybulsky MI (2023) The multi-faceted role of NADPH in regulating inflammation in activated myeloid cells. Front Immunol 14:

243. Everts B, Amiel E, Huang SC-C, Smith AM, Chang C-H, Lam WY, Redmann V, Freitas TC, Blagih J, van der Windt GJW, Artyomov MN, Jones RG, Pearce EL, Pearce EJ (2014) TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKε supports the anabolic demands of dendritic cell activation. Nat Immunol 15:323–332. https://doi.org/10.1038/ni.2833

244. Carroll RG, Zasłona Z, Galván-Peña S, Koppe EL, Sévin DC, Angiari S, Triantafilou M, Triantafilou K, Modis LK, O'Neill LA (2018) An unexpected link between fatty acid synthase and cholesterol synthesis in proinflammatory macrophage activation. J Biol Chem 293:5509–5521. https://doi.org/10.1074/jbc.RA118.001921

245. Gagnon E, Duclos S, Rondeau C, Chevet E, Cameron PH, Steele-Mortimer O, Paiement J, Bergeron JJM, Desjardins M (2002) Endoplasmic Reticulum-Mediated Phagocytosis Is a Mechanism of Entry into Macrophages. Cell 110:119–131. https://doi.org/10.1016/S0092-8674(02)00797-3

246. Ecker J, Liebisch G, Englmaier M, Grandl M, Robenek H, Schmitz G (2010) Induction of fatty acid synthesis is a key requirement for phagocytic differentiation of human monocytes. Proc Natl Acad Sci 107:7817–7822. https://doi.org/10.1073/pnas.0912059107

247. Kahlenberg JM, Carmona-Rivera C, Smith CK, Kaplan MJ (2013) Neutrophil Extracellular Trap–Associated Protein Activation of the NLRP3 Inflammasome Is Enhanced in Lupus Macrophages. J Immunol 190:1217–1226. https://doi.org/10.4049/jimmunol.1202388

248. Peng L, Tian H, Lu Y, Jia K, Ran J, Tao Q, Li G, Wan C, Ye C, Veldhuizen EJA, Chen H, Fang R (2022) Chicken cathelicidin-2 promotes NLRP3 inflammasome activation in macrophages. Vet Res 53:69. https://doi.org/10.1186/s13567-022-01083-4

249. Peng L, Lu Y, Tian H, Jia K, Tao Q, Li G, Wan C, Ye C, Veldhuizen EJA, Chen H, Fang R (2022) Chicken cathelicidin-2 promotes IL-1β secretion via the NLRP3 inflammasome pathway and serine proteases activity in LPS-primed murine neutrophils. Dev Comp Immunol 131:104377. https://doi.org/10.1016/j.dci.2022.104377

250. Boyman O, Sprent J (2012) The role of interleukin-2 during homeostasis and activation of the immune system. Nat Rev Immunol 12:180–190. https://doi.org/10.1038/nri3156

251. Malek TR (2008) The Biology of Interleukin-2. Annu Rev Immunol 26:453–479. https://doi.org/10.1146/annurev.immunol.26.021607.090357

252. Sadlack B, Merz H, Schorle H, Schimpl A, Feller AC, Horak I (1993) Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell 75:253–261. https://doi.org/10.1016/0092-8674(93)80067-O

253. Zhou L, Chu C, Teng F, Bessman NJ, Goc J, Santosa EK, Putzel GG, Kabata H, Kelsen JR, Baldassano RN, Shah MA, Sockolow RE, Vivier E, Eberl G, Smith KA, Sonnenberg GF (2019) Innate lymphoid cells support regulatory T cells in the intestine through interleukin-2. Nature 568:405–409. https://doi.org/10.1038/s41586-019-1082-x

254. Bachmann MF, Oxenius A (2007) Interleukin 2: from immunostimulation to immunoregulation and back again. EMBO Rep 8:1142–1148. https://doi.org/10.1038/sj.embor.7401099

255. Jarry A, Bossard C, Bou-Hanna C, Masson D, Espaze E, Denis MG, Laboisse CL (2008) Mucosal IL-10 and TGF-beta play crucial roles in preventing LPS-driven, IFN-gamma-mediated epithelial damage in human colon explants. J Clin Invest 118:1132–1142. https://doi.org/10.1172/JCI32140

256. Ma X, Shin Y-J, Jang H-M, Joo M-K, Yoo J-W, Kim D-H (2021) Lactobacillus rhamnosus and Bifidobacterium longum alleviate colitis and cognitive impairment in mice by

regulating IFN-γ to IL-10 and TNF-α to IL-10 expression ratios. Sci Rep 11:20659. https://doi.org/10.1038/s41598-021-00096-x

257. Ala Y, Pasha MK, Rao RN, Komaravalli PL, Jahan P (2015) Association of IFN-γ : IL-10 Cytokine Ratio with Nonsegmental Vitiligo Pathogenesis. Autoimmune Dis 2015:e423490. https://doi.org/10.1155/2015/423490

258. Jamil B, Shahid F, Hasan Z, Nasir N, Razzaki T, Dawood G, Hussain R (2007) Interferonγ/IL10 ratio defines the disease severity in pulmonary and extra pulmonary tuberculosis. Tuberculosis 87:279–287. https://doi.org/10.1016/j.tube.2007.03.004

259. Coorens M, Banaschewski BJH, Baer BJ, Yamashita C, van Dijk A, Haagsman HP, Veldhuizen RAW, Veldhuizen EJA (2017) Killing of Pseudomonas aeruginosa by Chicken Cathelicidin-2 Is Immunogenically Silent, Preventing Lung Inflammation In Vivo. Infect Immun 85:10.1128/iai.00546-17. https://doi.org/10.1128/iai.00546-17

260. Izcue A, Coombes JL, Powrie F (2009) Regulatory Lymphocytes and Intestinal Inflammation. Annu Rev Immunol 27:313–338. https://doi.org/10.1146/annurev.immunol.021908.132657

261. Tau G, Rothman P (1999) Biologic functions of the IFN-γ receptors. Allergy 54:1233. https://doi.org/10.1034/j.1398-9995.1999.00099.x

262. Kundu SD, Lee C, Billips BK, Habermacher GM, Zhang Q, Liu V, Wong LY, Klumpp DJ, Thumbikat P (2008) The toll-like receptor pathway: A novel mechanism of infection-induced carcinogenesis of prostate epithelial cells. The Prostate 68:223–229. https://doi.org/10.1002/pros.20710

263. Li Z-X, Li Q-Y, Qiao J, Lu C-Z, Xiao B-G (2009) Granulocyte-colony stimulating factor is involved in low-dose LPS-induced neuroprotection. Neurosci Lett 465:128–132. https://doi.org/10.1016/j.neulet.2009.08.069

264. Luhr M, Szalai P, Engedal N (2018) The Lactate Dehydrogenase Sequestration Assay — A Simple and Reliable Method to Determine Bulk Autophagic Sequestration Activity in Mammalian Cells. J Vis Exp JoVE. https://doi.org/10.3791/57971

265. Wu BC, Lee AH-Y, Hancock REW (2017) Mechanisms of the Innate Defense Regulator Peptide-1002 Anti-Inflammatory Activity in a Sterile Inflammation Mouse Model. J Immunol 199:3592–3603. https://doi.org/10.4049/jimmunol.1700985

10 Own scientific publications

Publications related to the topic of the present thesis

Full text papers in peer-reviewed journals:

Sebők C, Tráj P, Vörösházi J, Mackei M, Papp M, Gálfi P, Neogrády Z, Mátis G (2021) **Two Sides to Every Question: Attempts to Activate Chicken Innate Immunity in 2D and 3D Hepatic Cell Cultures**. Cells 10:1910. https://doi.org/10.3390/cells10081910

Sebők C, Walmsley S, Tráj P, Mackei M, Vörösházi J, Petrilla J, Kovács L, Kemény Á, Neogrády Z, Mátis G (2022) **Immunomodulatory effects of chicken cathelicidin-2 on a primary hepatic cell co-culture model**. PLOS ONE 17:e0275847. https://doi.org/10.1371/journal.pone.0275847

Sebők C, Tráj P, Mackei M, Márton RA, Vörösházi J, Kemény Á, Neogrády Z, Mátis G (2023) **Modulation of the immune response by the host defense peptide IDR-1002 in chicken hepatic cell culture**. Sci Rep 13:14530. https://doi.org/10.1038/s41598-023-41707-z

Mátis G, Tráj P, Hanyecz V, Mackei M, Márton RA, Vörösházi J, Kemény Á, Neogrády Z, Sebők C (2024) **Immunomodulatory properties of chicken cathelicidin-2 investigated on an ileal explant culture**. Vet Res Commun 48:2527–2535. https://doi.org/10.1007/s11259-024-10428-7

Sebők C, Márton Rege A, Mackei M, Neogrády Z, Mátis G (2024) **Az antimikrobiális peptidek mint a fertőző betegségek elleni küzdelem új eszközei. Antimicrobial peptides as new tools to combat infectious diseases**. MÁL. 2024.03.181-191. https://doi.org/10.56385/

Mátis G, Tráj P, Hanyecz V, Mackei M, Márton RA, Vörösházi J, Kemény Á, Neogrády Z, Sebők C (2025) **Immunomodulatory effects of the Host Defense Peptide IDR-1002 investigated on chicken ileal explants. – Under review**

Poster presentations at international conferences

Sebők C, Walmsley S, Tráj P, Mackei M, Vörösházi J, Kemény Á, Neogrády Zs, Mátis G (2022) **Immunomodulatory Effects of Chicken Cathelicidin-2 on a Primary Hepatic Cell Co-Culture Model,** 15th International Scientific Conference On Probiotics, Prebiotics, Gut Microbiota And Health, Pozsony, 2022.06.27-30.

Sebők, C; Tráj, P; Mackei, M; Kemény, Á; Neogrády, Zs; Mátis, G: **The immunomodulatory action of the antimicrobial peptide IDR-1002 in a hepatic cell culture model of chicken origin**, Proceedings of the 13th International Veterinary Immunology Symposium, Kruger National Park, South Africa. 17-21 November 2023, page 86, abstract 123., 2023

Oral presentations at Hungarian national conferences

Kulcsár A, Sebők C, Mátis G, Talapka P, Hatala P, Petrilla J, Fébel H, Neogrády Zs (2017) **Az inzulin és a glukagon jelpálya különböző takarmányozási faktorok segítségével történő szabályozása broilercsirkében**, Akadémiai Beszámolók 2017. évi 44. füzet.

Sebők C, Orbán K, Mackei M, Vörösházi J, Neogrády Zs, Mátis G (2018) **A chicken heterophil peptide 1 (CHP-1) gyulladáscsökkentő hatásának vizsgálata csirke eredetű májsejt-tenyészetek**en, Akadémiai Beszámolók 2019. évi 46. füzet.

Sebők C, Vörösházi J, Mackei M, Tráj P, Szentgyörgyi Á, Neogrády Zs és Mátis G (2019) **Bakteriális sejtfalkomponensek hatásainak vizsgálata két-, illetve háromdimenziós csirke eredetű májsejttenyészeteken**, Akadémiai Beszámolók 2020. évi 47. füzet

Sebők C, Walmsley S, Tráj P, Mackei M, Vörösházi J, Kemény Á, Márton R A, Neogrády Zs, Mátis G (2022) **A cathelicidin-2 immunmoduláló hatásának vizsgálata csirke eredetű májsejttenyészeteken**, Akadémiai Beszámolók 2021. évi 48. füzet.

Sebők C, Tráj P, Mackei M, Márton R A, Vörösházi J, Kemény Á, Neogrády Zs és Mátis G (2023) **Az IDR-1002 immunmodulátor tulajdonságának vizsgálata csirke eredetű májsejt – nem-parenchimális sejt ko-kultúrán**, Akadémiai Beszámolók 2022. évi 49. füzet

Sebők C, Tráj P, Mackei M, Márton R A, Vörösházi J, Kemény Á, Neogrády Zs és Mátis G (2024) **Az IDR-1002 ntimikrobiális peptid gyulladásos folyamatokra kifejtett hatásainak vizsgálata csirke eredetű vékonybél explantokon**, Akadémiai Beszámolók 2023. évi 50. füzet

Publications not related to the topic of the present dissertation

Mackei M, Vörösházi J, Sebők C, Neogrády Z, Mátis G, Jerzsele Á (2020) **Fermented Wheat Germ Extract as a Redox Modulator: Alleviating Endotoxin-Triggered Oxidative Stress in Primary Cultured Rat Hepatocytes**. Oxid Med Cell Longev 2020:3181202. https://doi.org/10.1155/2020/3181202

Mackei M, Sebők C, Vöröházi J, Tráj P, Mackei F, Oláh B, Fébel H, Neogrády Z, Mátis G (2023) **Detrimental consequences of tebuconazole on redox homeostasis and fatty acid profile of honeybee brain**. Insect Biochem Mol Biol 159:103990. https://doi.org/10.1016/j.ibmb.2023.103990

Mackei M, Mátis G, Molnár A, Sebők C, Vörösházi J, Pál L, Dublecz K, Husvéth F, Neogrády Z (2021) **The relationship between small heat shock proteins and redox homeostasis during acute heat stress in chickens**. J Therm Biol 100:103040. https://doi.org/10.1016/j.jtherbio.2021.103040

Tráj P, Sebők C, Mackei M, Kemény Á, Farkas O, Kákonyi Á, Kovács L, Neogrády Z, Jerzsele Á, Mátis G (2023) **Luteolin: A Phytochemical to Mitigate S. Typhimurium Flagellin-Induced Inflammation in a Chicken In Vitro Hepatic Model.** Animals 13:1410. https://doi.org/10.3390/ani13081410

Tráj P, Herrmann EM, Sebők C, Vörösházi J, Mackei M, Gálfi P, Kemény Á, Neogrády Z, Mátis G (2022) **Protective effects of chicoric acid on polyinosinic-polycytidylic acid exposed chicken hepatic cell culture mimicking viral damage and inflammation.** Vet Immunol Immunopathol 250:110427. https://doi.org/10.1016/j.vetimm.2022.110427

Hatala P, Lajos A, Mackei M, Sebők C, Tráj P, Vörösházi J, Neogrády Z, Mátis G (2023) Feline **Uroepithelial Cell Culture as a Novel Model of Idiopathic Cystitis: Investigations on the Effects of Norepinephrine on Inflammatory Response, Oxidative Stress, and Barrier Function**. Vet Sci 10:132. https://doi.org/10.3390/vetsci10020132

Petrilla J, Mátis G, Mackei M, Kulcsár A, Sebők C, Papp M, Gálfi P, Fébel H, Huber K, Neogrády Z (2022) **Modulation of Hepatic Insulin and Glucagon Signaling by Nutritional Factors in Broiler Chicken**. Vet Sci 9:103. https://doi.org/10.3390/vetsci9030103

Márton RA, Sebők C, Mackei M, Tráj P, Vörösházi J, Kemény Á, Neogrády Z, Mátis G (2024) **Cecropin A: investigation of a host defense peptide with multifaceted immunomodulatory activity in a chicken hepatic cell culture**. Front Vet Sci 11:1337677. https://doi.org/10.3389/fvets.2024.1337677

Márton RA, Sebők C, Mackei M, Tráj P, Vörösházi J, Kemény Á, Neogrády Z, Mátis G (2024) **Pap12-6: A host defense peptide with potent immunomodulatory activity in a chicken hepatic cell culture.** PLOS ONE 19:e0302913. https://doi.org/10.1371/journal.pone.0302913

Vörösházi J, Mackei M, Sebők C, Tráj P, Márton RA, Horváth DG, Huber K, Neogrády Z, Mátis G (2024) **Investigation of the effects of T-2 toxin in chicken-derived three-dimensional hepatic cell cultures.** Sci Rep 14:1195. https://doi.org/10.1038/s41598-024-51689-1

Hatala P, Sebők C, Mackei M, Kárpáti K, Gálfi P, Neogrády Z, Mátis G (2023) **Molecular effects of intermittent stress on primary feline uroepithelial cell culture as an in vitro model of feline idiopathic cystitis.** Front Vet Sci 10:1258375. https://doi.org/10.3389/fvets.2023.125837

Supervision of DVM theses:

Szentgyörgyi Ákos: **Comparison of the effects of bacterial cell wall components on two- and three-dimensional chicken liver cell cultures**. TDK thesis. Supervisors: Csilla Sebők and Júlia Vörösházi, Budapest, 2020.

Walmsley, Stephanie: **Immunomodulatory role of cathelicidin-2 in chicken primary hepatocyte - non-parenchymal cell co-cultures.** TDK thesis. Supervisors: Csilla Sebők and Zsuzsanna Neogrády, Budapest, 2021.

Vágvölgyi Petra: **Immunomodulatory and antioxidant effects of IDR-1002 investigated on a hepatic co-culture of chicken origin**. TDK thesis. Supervisors: Csilla Sebők and Gábor Mátis, Budapest, 2022.

Ujházy András: **Effects of the antimicrobial peptide IDR-1002 on inflammatory processes in the intestinal mucosa of chicken**. TDK thesis. Supervisor: Csilla Sebők, Budapest, 2023.

Hanyecz Viktória: **Investigation of the immunomodulatory effects of chicken cathelicidin-2 on chicken intestinal explants.** TDK thesis. Supervisor: Csilla Sebők, Budapest, 2023.

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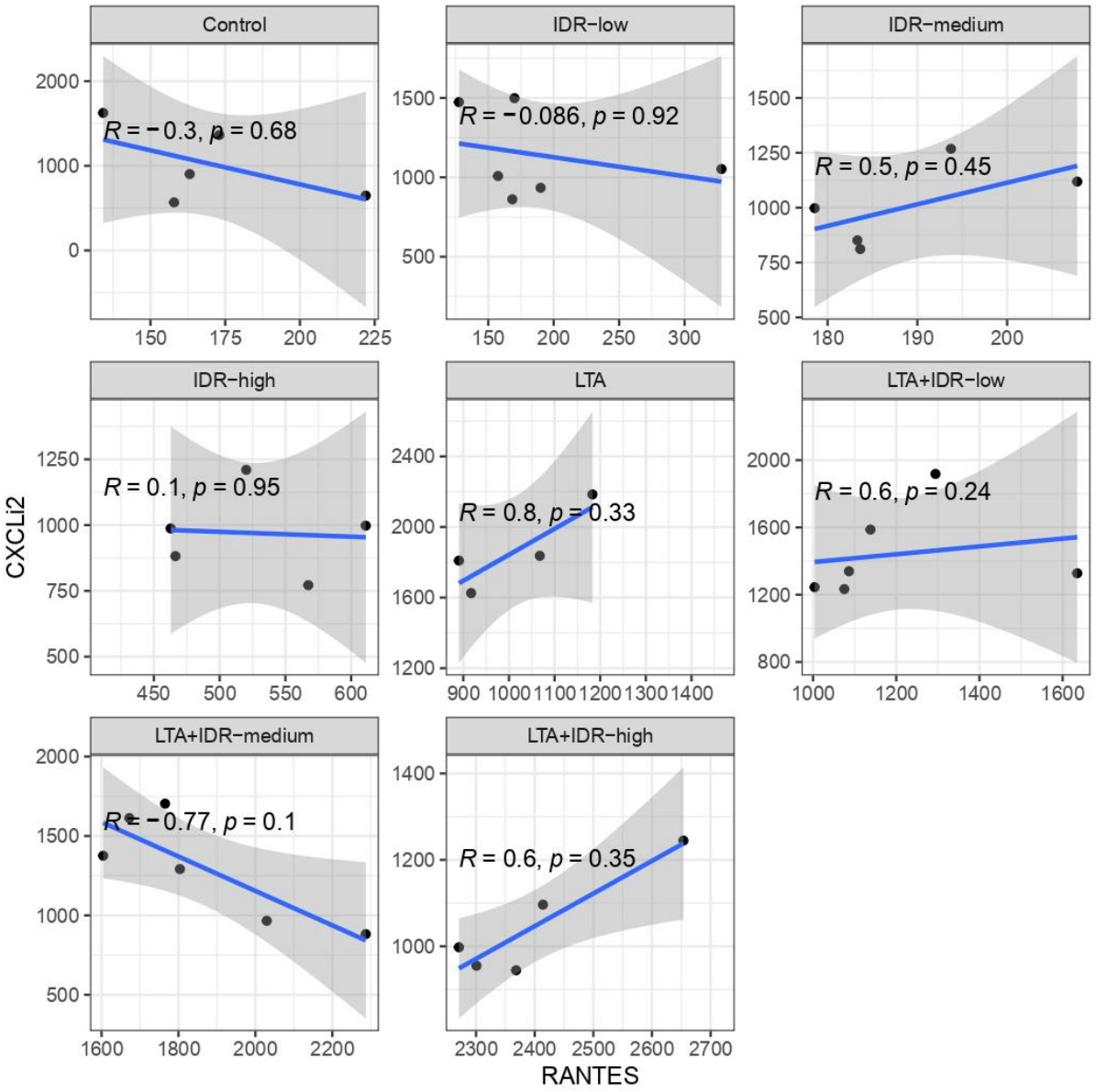
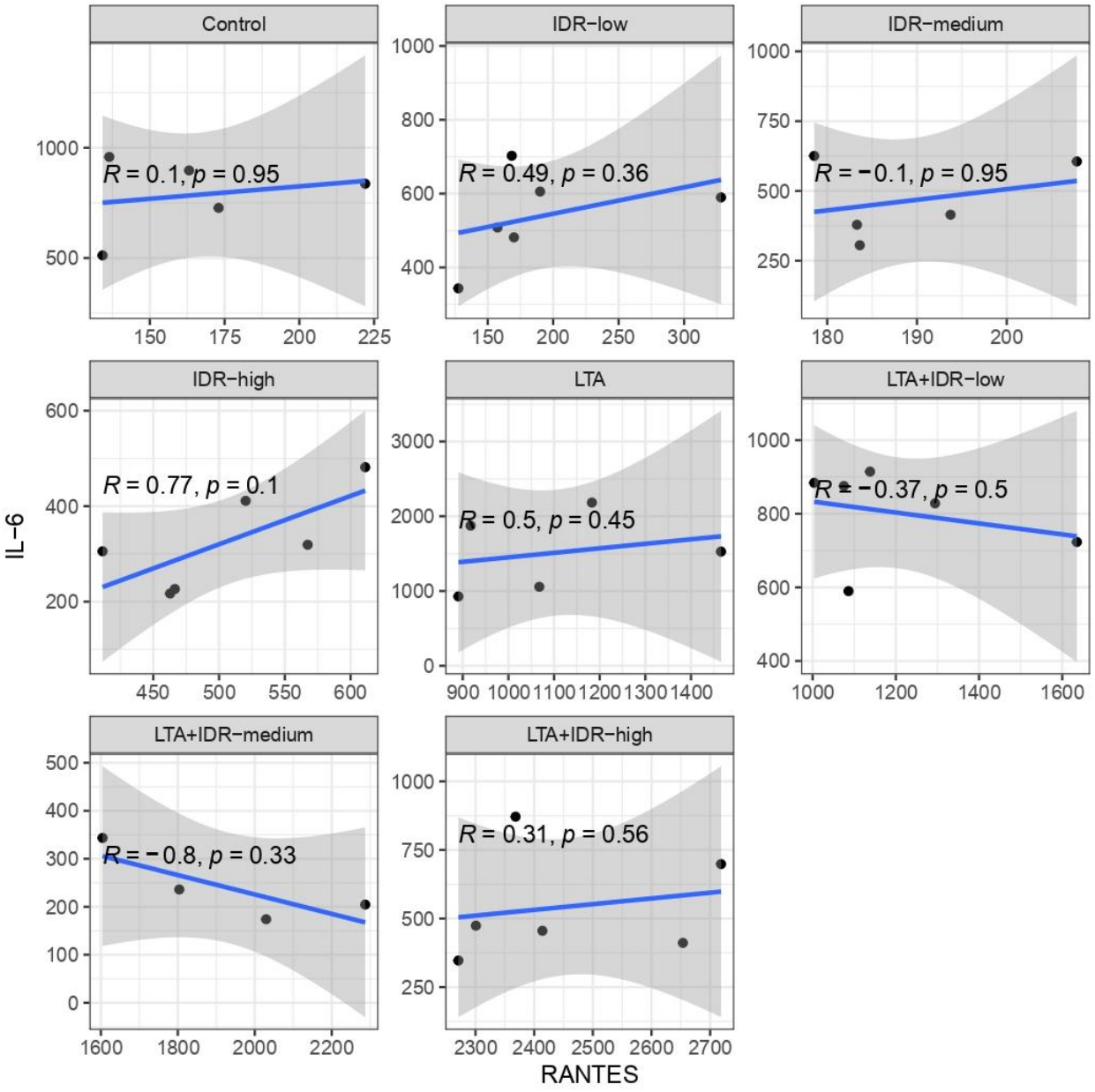
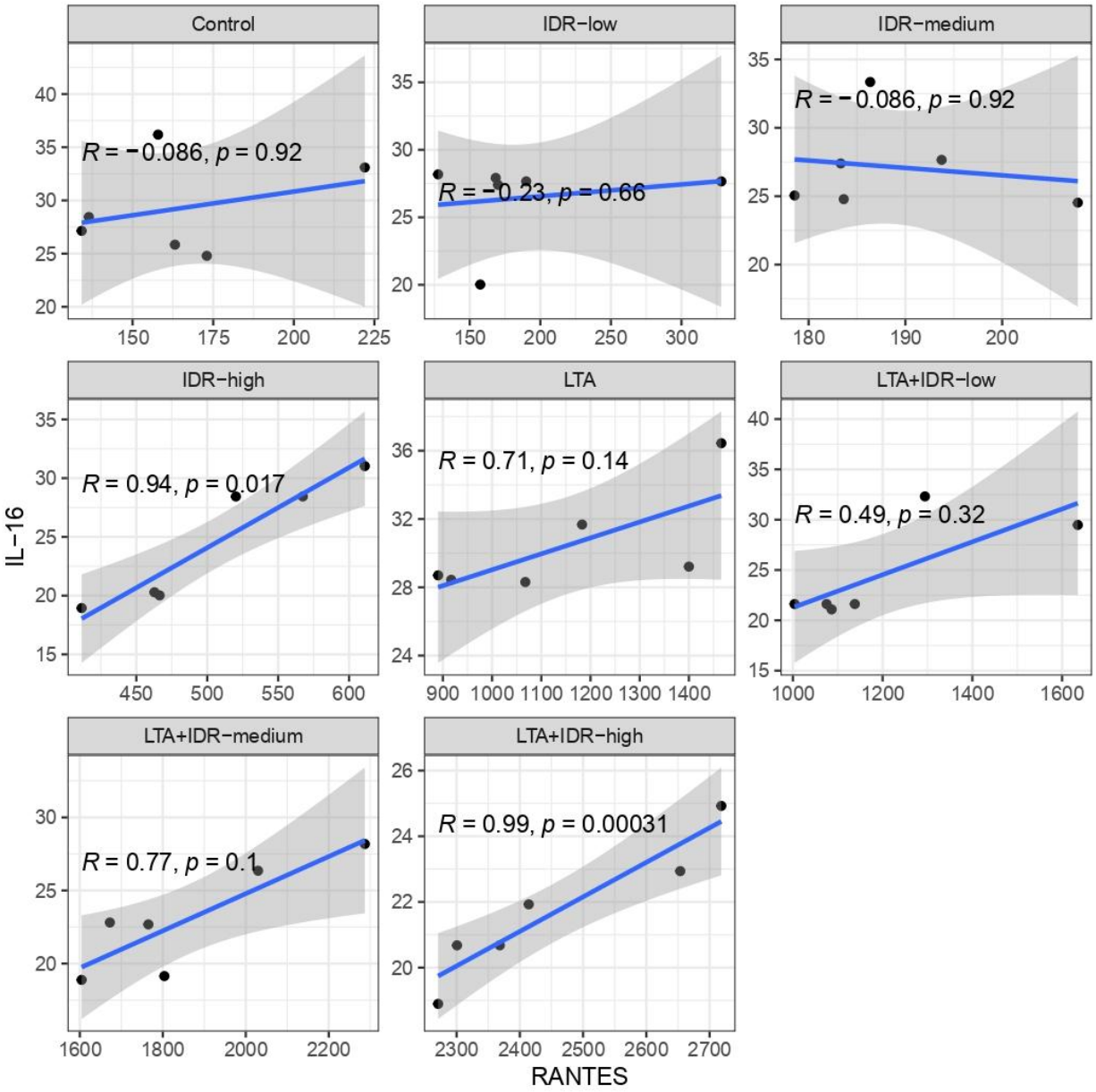
12 Supplementary material



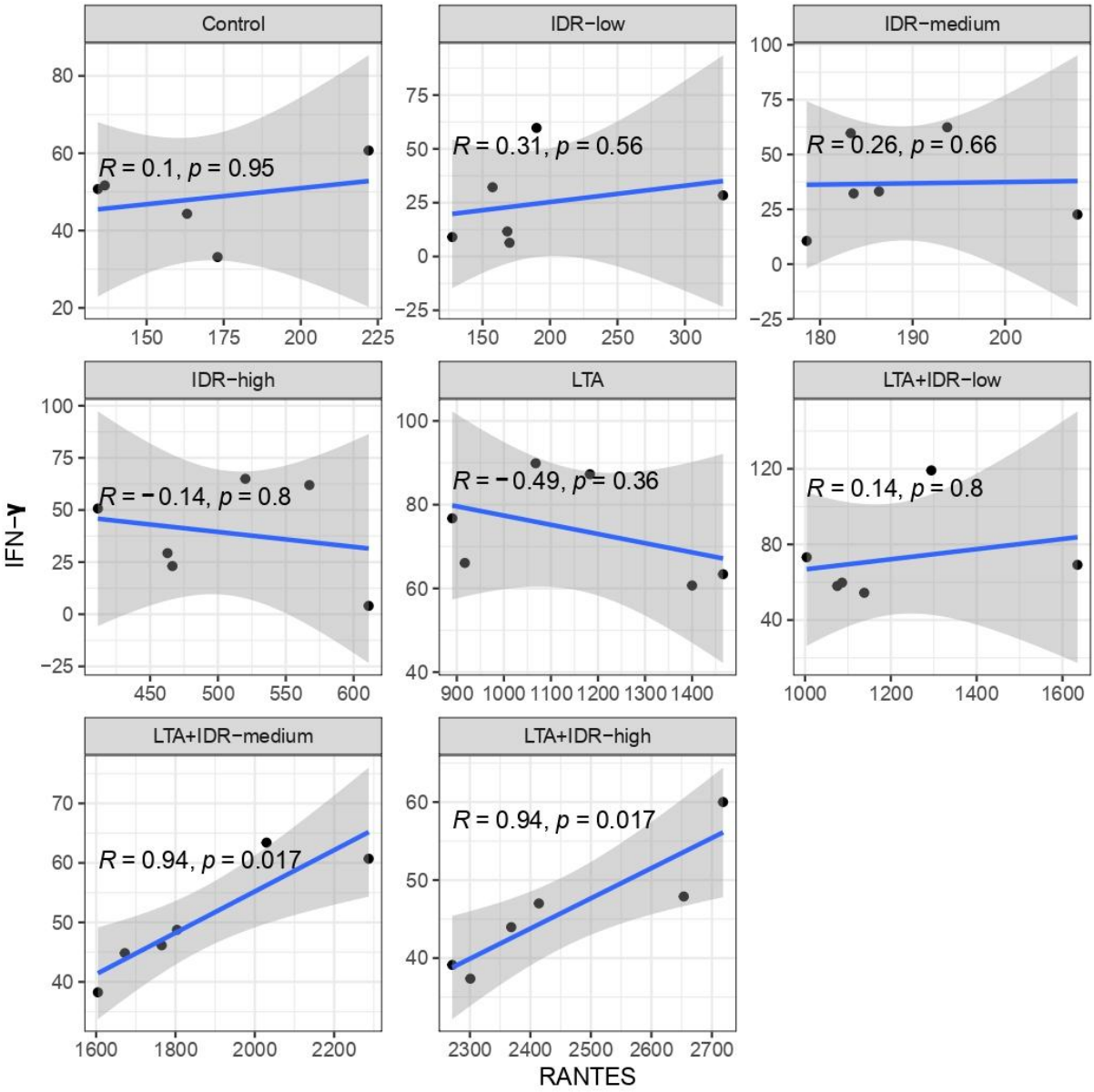
Supplementary Figure 1: Scatterplot showing the association between RANTES and CXCLi2 values for each treatment group. Linear regression lines (with 95% confidence band) and Spearman correlation coefficients with p-values are displayed on each subplot for every treatment group. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 50 µg/mL lipotheichoic acid from Staphylococcus aureus (n = 6/group). Cell cultures in Control group received none of the treatments.



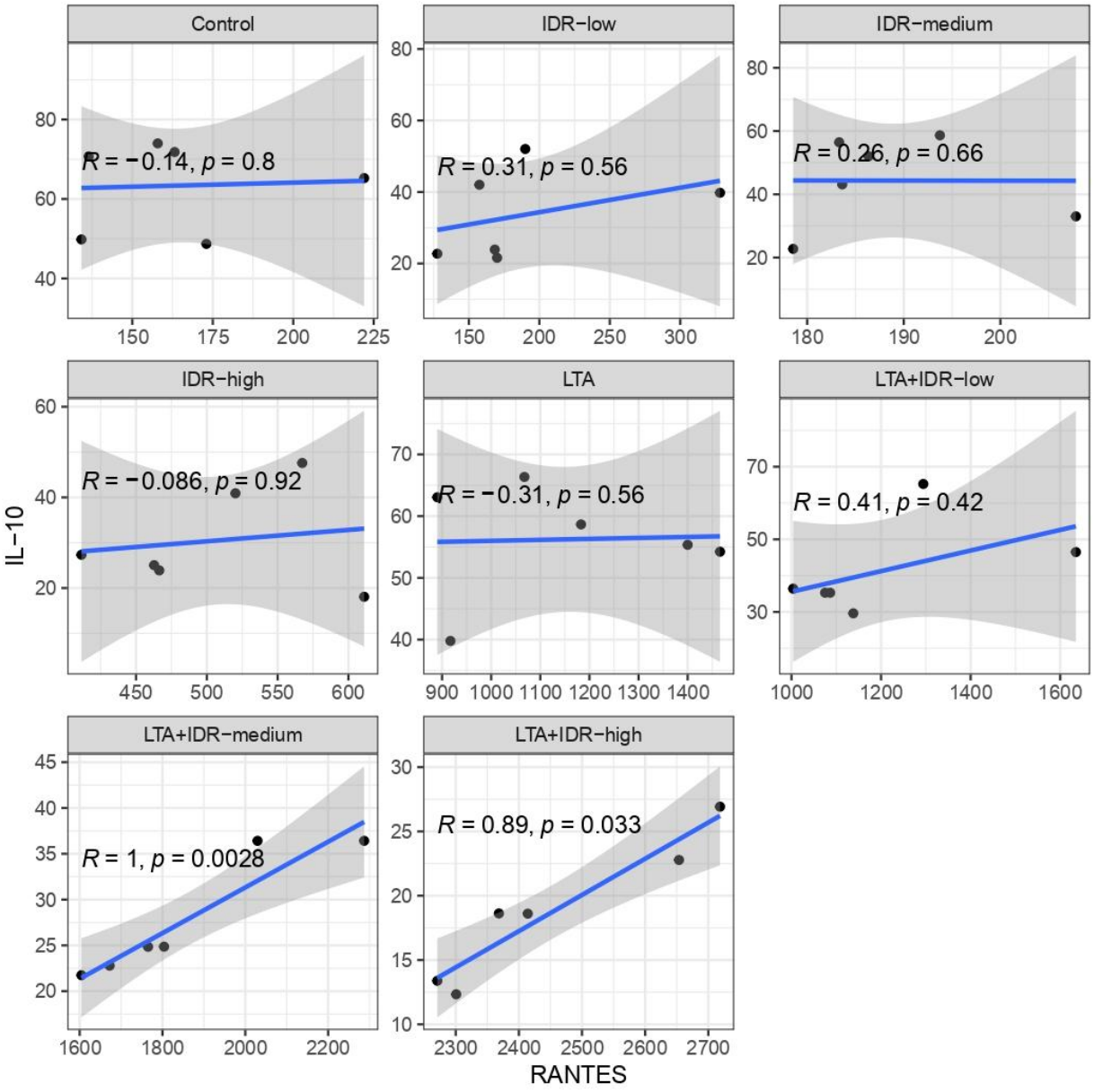
Supplementary Figure 2: Scatterplot showing the association between RANTES and Interleukin (IL)-6 values for each treatment group. Linear regression lines (with 95% confidence band) and Spearman correlation coefficients with p-values are displayed on each subplot for every treatment group. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 50 µg/mL lipotheichoic acid from Staphylococcus aureus (n = 6/group). Cell cultures in Control group received none of the treatments.



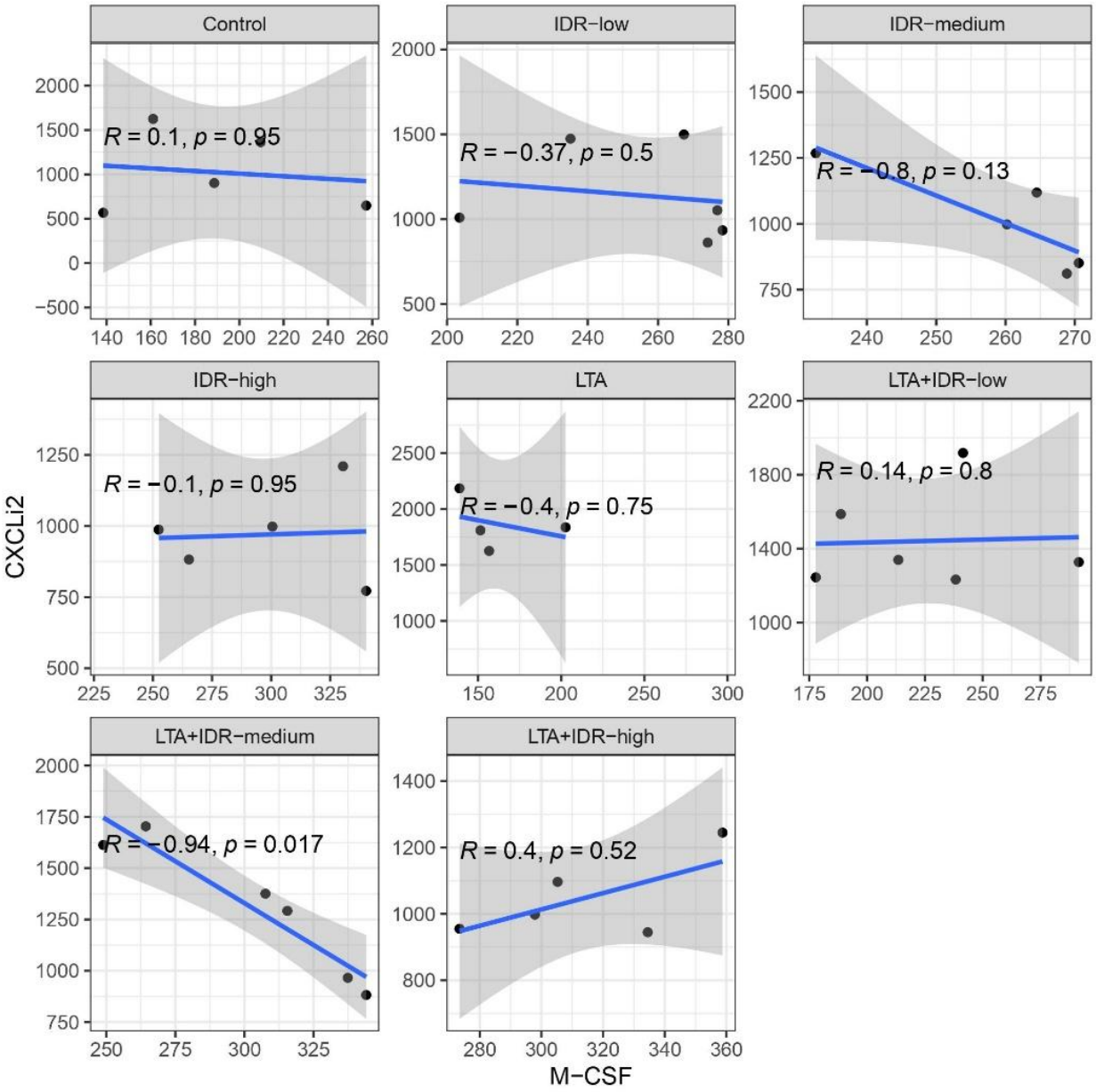
Supplementary Figure 3: Scatterplot showing the association between RANTES and Interleukin (IL)-16 values for each treatment group. Linear regression lines (with 95% confidence band) and Spearman correlation coefficients with p-values are displayed on each subplot for every treatment group. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 50 µg/mL lipotheichoic acid from Staphylococcus aureus (n = 6/group). Cell cultures in Control group received none of the treatments.



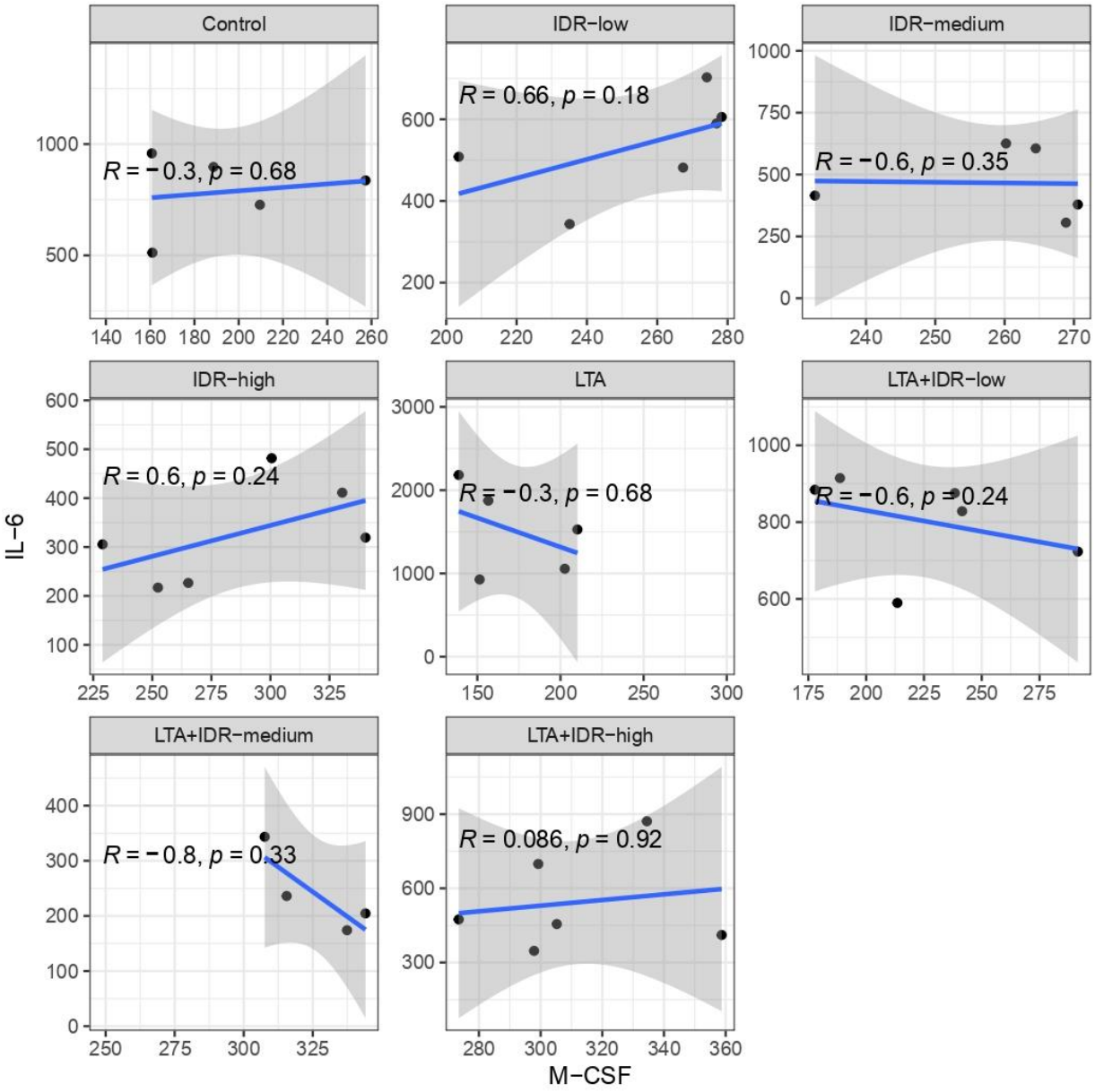
Supplementary Figure 4: Scatterplot showing the association between RANTES and Interferon (IFN)-γ values for each treatment group. Linear regression lines (with 95% confidence band) and Spearman correlation coefficients with p-values are displayed on each subplot for every treatment group. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 50 µg/mL lipotheichoic acid from Staphylococcus aureus (n = 6/group). Cell cultures in Control group received none of the treatments.



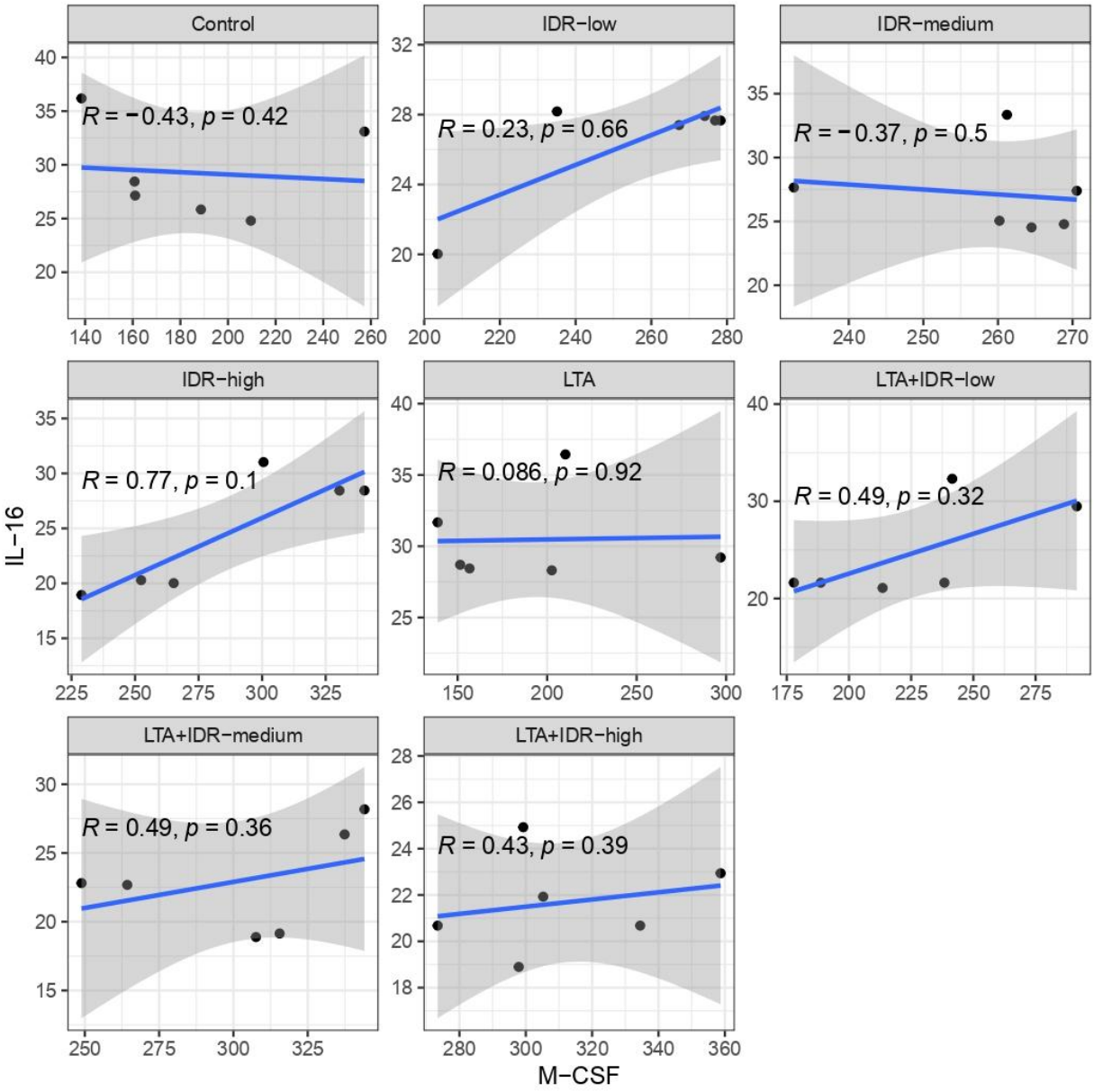
Supplementary Figure 5: Scatterplot showing the association between RANTES and Interleukin (IL)-10 values for each treatment group. Linear regression lines (with 95% confidence band) and Spearman correlation coefficients with p-values are displayed on each subplot for every treatment group. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 50 µg/mL lipotheichoic acid from Staphylococcus aureus (n = 6/group). Cell cultures in Control group received none of the treatments.



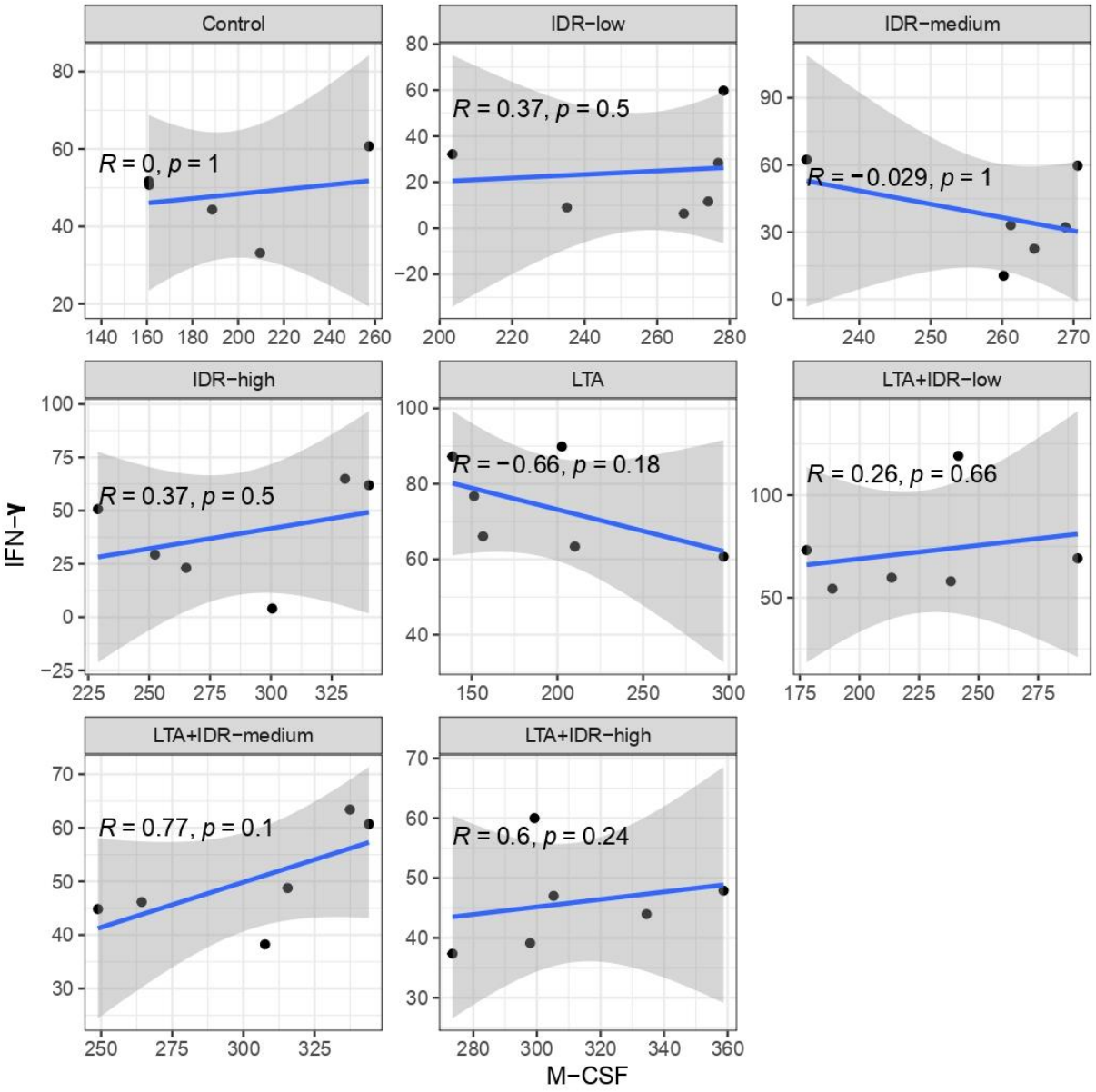
Supplementary Figure 6: Scatterplot showing the association between M-CSF and CXCLi2 values for each treatment group. Linear regression lines (with 95% confidence band) and Spearman correlation coefficients with p-values are displayed on each subplot for every treatment group. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 50 µg/mL lipotheichoic acid from Staphylococcusaureus (n = 6/group). Cell cultures in Control group received none of the treatments.



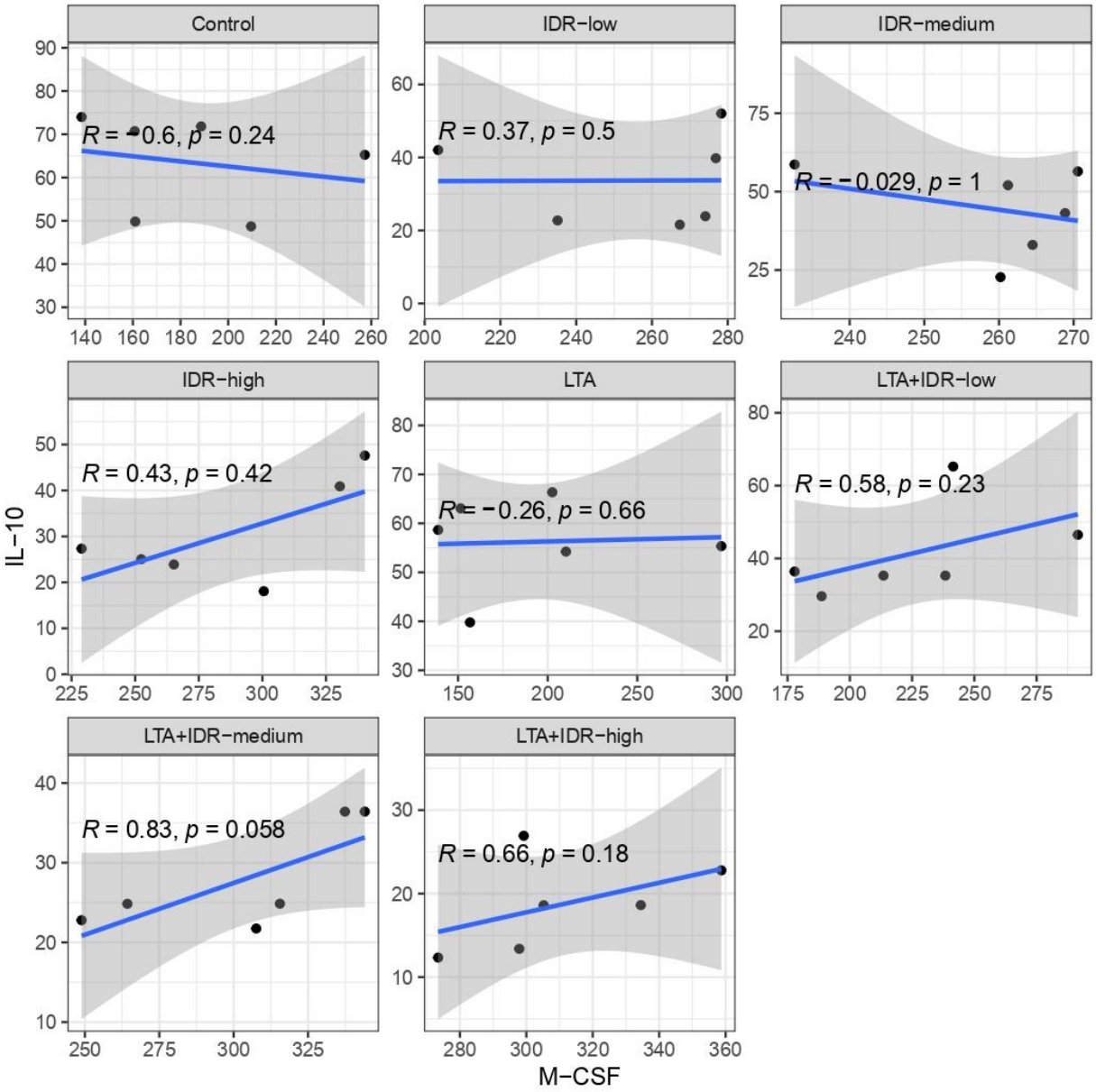
Supplementary Figure 7: Scatterplot showing the association between M-CSF and Interleukin (IL)-6 values for each treatment group. Linear regression lines (with 95% confidence band) and Spearman correlation coefficients with p-values are displayed on each subplot for every treatment group. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 50 µg/mL lipotheichoic acid from Staphylococcus aureus (n = 6/group). Cell cultures in Control group received none of the treatments.



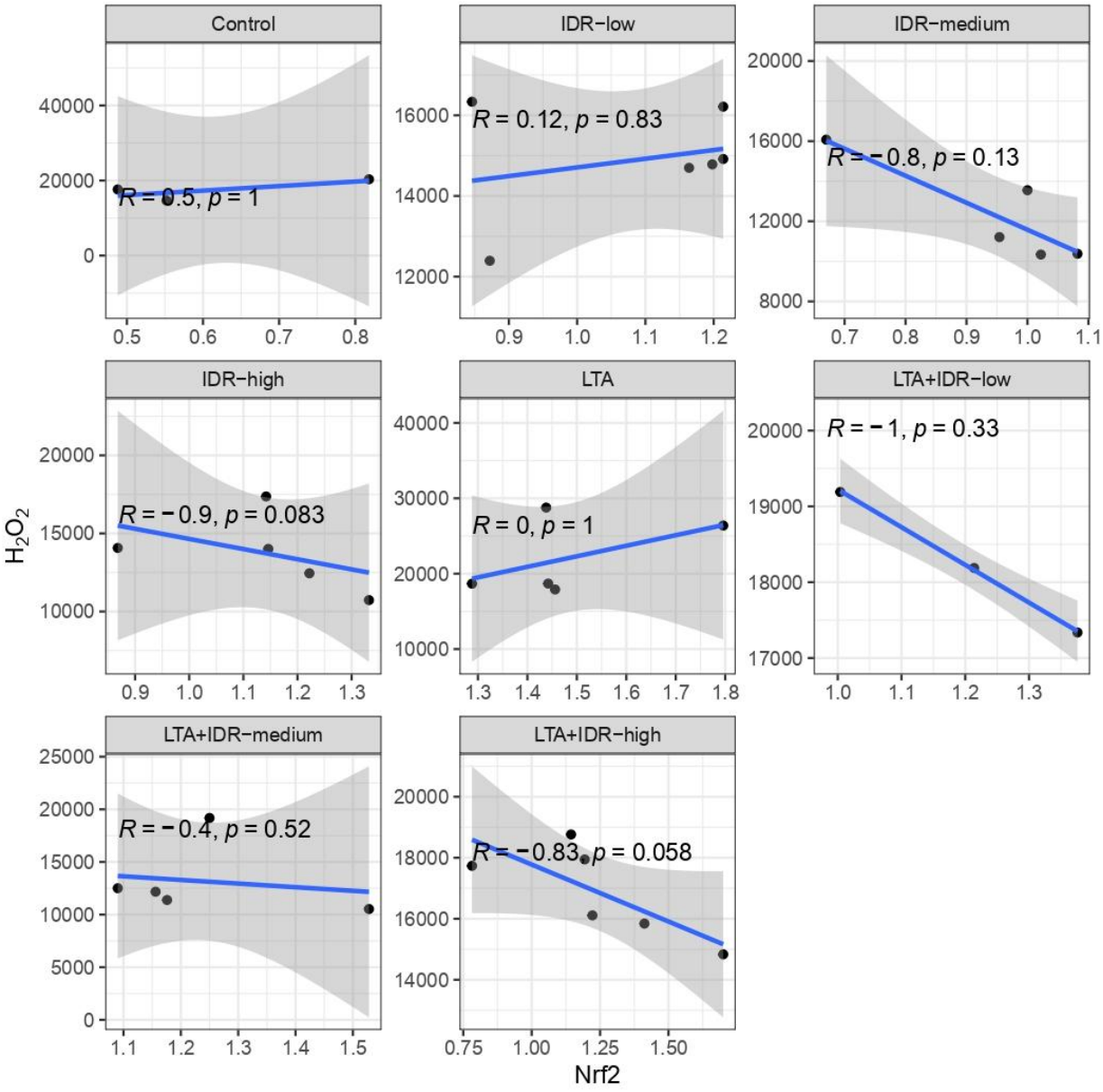
Supplementary Figure 8: Scatterplot showing the association between M-CSF and Interleukin (IL)-16 values for each treatment group. Linear regression lines (with 95% confidence band) and Spearman correlation coefficients with p-values are displayed on each subplot for every treatment group. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 50 µg/mL lipotheichoic acid from Staphylococcus aureus (n = 6/group). Cell cultures in Control group received none of the treatments.



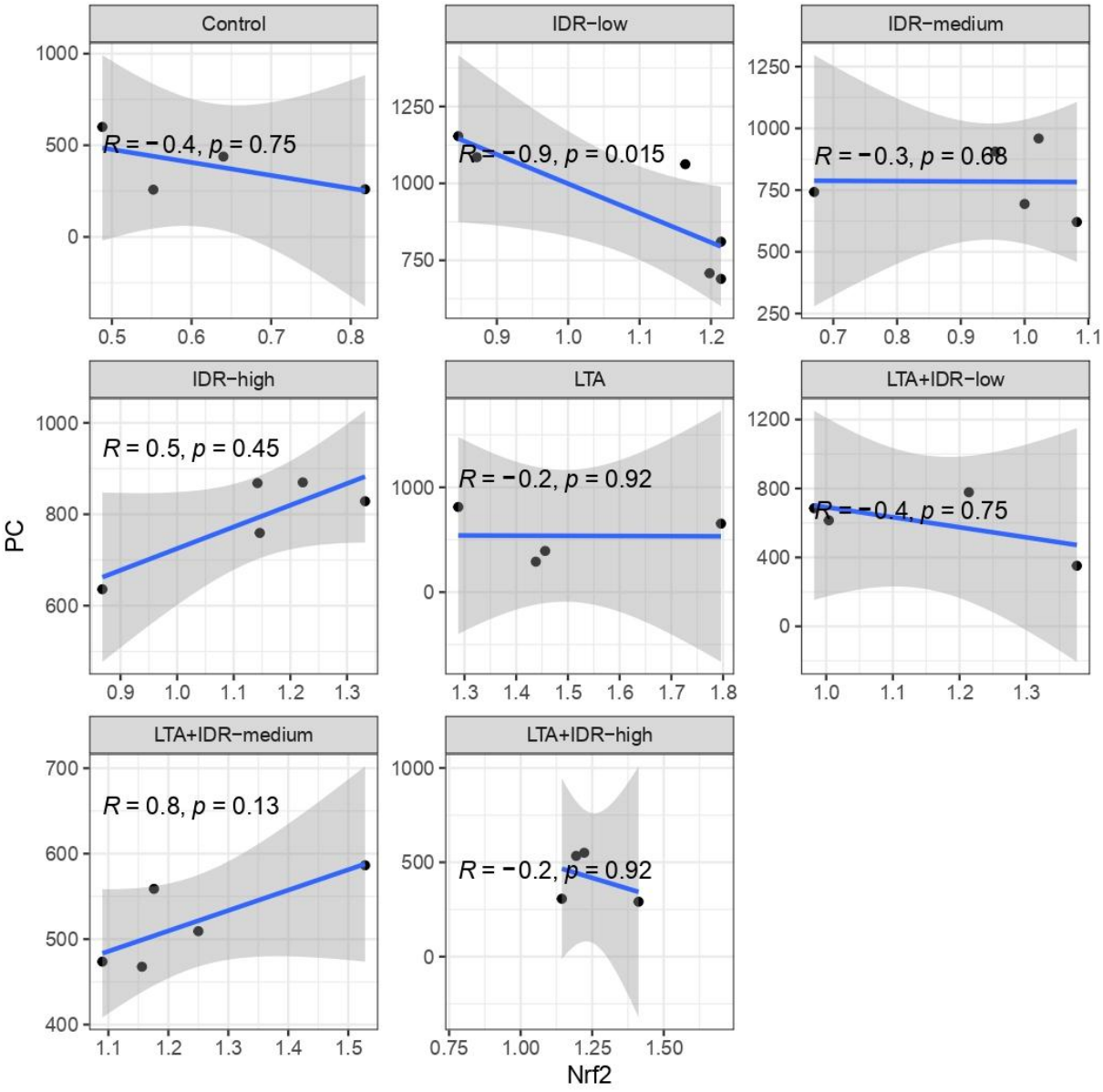
Supplementary Figure 9: Scatterplot showing the association between M-CSF and Interferon (IFN)-γ values for each treatment group. Linear regression lines (with 95% confidence band) and Spearman correlation coefficients with p-values are displayed on each subplot for every treatment group. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 50 µg/mL lipotheichoic acid from Staphylococcus aureus (n = 6/group). Cell cultures in Control group received none of the treatments.



Supplementary Figure 10: Scatterplot showing the association between M-CSF and Interleukin (IL)-10 values for each treatment group. Linear regression lines (with 95% confidence band) and Spearman correlation coefficients with p-values are displayed on each subplot for every treatment group. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 50 µg/mL lipotheichoic acid from Staphylococcus aureus (n = 6/group). Cell cultures in Control group received none of the treatments.



Supplementary Figure 11: Scatterplot showing the association between Nrf2 and H_2O_2 values for each treatment group. Linear regression lines (with 95% confidence band) and Spearman correlation coefficients with p-values are displayed on each subplot for every treatment group. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 50 µg/mL lipotheichoic acid from Staphylococcus aureus (n = 6/group). Cell cultures in Control group received none of the treatments.



Supplementary Figure 12: Scatterplot showing the association between Nrf2 and Protein Carbonyl (PC) values for each treatment group. Linear regression lines (with 95% confidence band) and Spearman correlation coefficients with p-values are displayed on each subplot for every treatment group. IDR-low = 10 µg/mL IDR-1002, IDR-medium = 30 µg/mL IDR-1002, IDR-high = 90 µg/mL IDR-1002, LTA = 50 µg/mL lipotheichoic acid from Staphylococcus aureus (n = 6/group). Cell cultures in Control group received none of the treatments.